UNIVERSITY OF LONDON THESIS

Degree PhD Year 2003 Name of Author ANAND

Arni

Characterization of $CD3^+CD4^-CD8^-$ (double negative) T cells in patients with systemic lupus erythematosus (SLE).

by

Arthi Anand

Department of Immunology
University College London Medical School

A thesis submitted for the degree of Doctor of Philosophy.
University of London 2003

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT.

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease characterized serologically by B cell hyperactivity and a panoply of autoantibodies against nuclear, cytoplasmic and cell surface antigens. It is thought that T cells are involved in this process and more recently it has been suggested that the $CD4^-$ $CD8^-$, i.e.double negative (DN) T cells, might be important.

As a start to understanding the contribution of DN T cells to disease pathogenesis in SLE, the percentages of DN T cells were determined and it was found that αβ but not γδ DNT cells were significantly increased in patients with SLE when compared to rheumatoid arthritis (RA) (autoimmune controls) and healthy controls.

To further establish their participation in the autoimmune reactions in SLE, the activation markers expressed by the DN T cells were examined. It was found that HLA-DR and CD69, and co-stimulatory molecules CD28 and CTLA-4 were all expressed by significantly higher percentages of DNT cells from patients with SLE, than those with RA or healthy controls (HC). More DN T cells from SLE patients were $CD45RA^+$ than from controls, while $CD45R0^+$ were reduced. DN T cells in patients with SLE also showed a more activated phenotype than their $CD4^+$/ $CD8^+$ counterparts.

To understand the functional significance in SLE DNT cells, the percentages of SLE αβ TCR^+ DN T cells containing intracellular IL-4, a Th2 cytokine was determined. Higher percentages of SLE αβ TCR DN^+ T cells contained IL-4 constitutively than RA or HC.

DN T cell populations from patients with SLE showed greater resistance to apoptosis in culture than the conventional CD4/$CD8^+$ cells and DN T cells from healthy controls. High Bcl-2/Bax ratios and higher levels of Bcl-x observed in the DN T cells from patients with SLE could explain their resistance to apoptosis compared to the conventional T cells and DN T cells from healthy controls.

CONTENTS.

TABLE OF CONTENTS.

CHAPTER 1
GENERAL INTRODUCTION

CHAPTER 5
CYTOKINE SECRETION BY SLE DN T CELLS

CHAPTER 6
APOPTOSIS IN SLE DN T CELLS

LIST OF FIGURES.

LIST OF TABLES.

ABBREVIATIONS.

α	alpha
αβ	alpha beta
ACR	American congress of Rheumatology
AICD	Activation induced cell death
AITD	Autoimmune thyroid disease
ALPS	Autoimmune lymphoproliferative syndrome
AMP	Adenosine mono phosphate
ANA	Anti nuclear antibody
AO	Acridine orange
APC	Antigen presenting cell
ARA	American Rheumatology Association
ATL	Adult T cell leukemia
BSA	Bovine serum albumin
C	Complement protein
Ca	Calcium
°C	Degree celsius
CD	Cluster of differentiation
CTLA-4	Cytotoxic T lymphocyte Associated Antigen 4
DN	Double negative
DNA	Deoxy-ribonucleic-acid
ds	Double stranded
ELISA	Enzyme Linked Immunosorbent Assay
F1	First generation
FACScan	Fluorescence activated cell scanner
Fc	Crystallizable fragment of the immunoglobulin molecule
FcR	Receptor for Fc region of immunoglobulin
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
FSC	Forward light scatter
γ	gamma
γδ	gamma delta
g	Gravity
G	Cell cycle growth phase
GBM	Glomerular Basement Membrane
GLU	L-Glutamine
HC	Healthy control
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN	Interferon
IL	Interleukin
kDa	KiloDalton
M	Mitosis
MFI	Mean Fluorescence Intensity
MHC	Major histocompatability complex
mg	Milligram

ml	Millilitre
μg	Microgram
μl	Microlitre
MoAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
NK	Natural Killer
NKT	Natural killer T cell
NMS	Normal mouse serum
NSAIDs	Non-steroidal anti-inflammatory drugs
NZB	New Zealand Black
NZW	New Zealand White
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed death 1 receptor
PHA	Phytohaemagglutinin
PKA	Protein kinase A
PMA	Phorbol myristic acetate
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Synthesis
SAP	Serum amyloid P
sd	Standard deviation
SLE	Systemic lupus erythematosus
SS	Single stranded
SSC	Side scatter (90° light scatter)
TCR	T cell receptor
Th	T helper
TNF-α	Tumour necrosis factor α
UV	Ultra Violet
Yaa	Y linked autoimmune accelerator

AKNOWLEDGEMENTS.

This work would not have been possible without the help and guidance of Professor Peter Lydyard, whose encyclopædic knowledge of immunology gave me an enthusiasm for the subject. My gratitude to Dr Mark Bodman Smith for teaching me the correct approach to practical bench work. A special thank you to Dr Gillian Dean for providing some of the data included in the chapter measuring IL-4 in double negative T cells. I am also deeply grateful to Professor David Isenberg for providing all the clinical blood samples, listening patiently to my results every week during the Monday evening meetings and also for kindly providing the financial support I needed to complete this work.

To everyone in the Bloomsbury Rheumatology unit I must say a huge "Thank You" for the enormous help in finding clinical samples for me. To Avery and Abigail, the nurses, for putting up with my constant interruptions into their busy Thursday morning clinics, pestering for more samples, a mere thank you will not suffice. Special mention must also go to Jonathan Tyrell-Price for patiently collecting blood for all the research studies and all the 'discussions' which saw me through the PhD.

The whole of the Department of Immunology at UCLMS deserves my gratitude, for stoically allowing me to borrow consumables and reagents, and generously teaching me new techniques. They made it all fun. Special Thanks to Dr. Yvette Latchmann and Dr Peter Delves for going through the thesis and their helpful and encouraging suggestions.

To the British Council Overseas Research Scholarship Awards scheme for Universities and Colleges in the United Kingdom and The Arthritis Research Campaign for providing the funding which enabled me to pursue my PhD degree, I am deeply grateful.

My parents, who put up with my endless reading and questions, must take credit for instilling in me the importance of knowledge, and giving me a good education. Special thanks also to Ms.V. Bhuvaneshwari for nurturing my interest in biology.

To Anand, my husband, for supporting me through the highs and lows of the PhD years, patiently and generously, and the two years spent away from his son. Thank you, I could not have done it without you. Finally to my son, Rakshith, for always putting a smile on my face and making me see the exciting possibilities of every day.

Chapter 1.

General Introduction.

1.1: Systemic lupus erythematosus.

Systemic lupus erythematosus is considered to be the prototypic systemic autoimmune rheumatic disease (Kotzin, 1996). It is a syndrome of complex multifactorial origin characterised by inflammation and has the potential to involve any of the body's organs or systems (Isenberg, 1993). The American Congress of Rheumatology (ACR) revised classification criteria were originally formulated to classify the heterogenous population of SLE patients: it is widely used as a diagnostic standard. A patient can be diagnosed with SLE if they serially or simultaneously exhibit four of these criteria. This classification 'set' gives a correct definition of SLE with 95% sensitivity and 85% specificity (Hahn, 1998). The criteria are summarised in table 1.1:

- Malar rash
- Discoid rash
- Photosensitivity
- Oral ulcers
- Arthritis
- Serositis
--pleuritis
or pericarditis
- Renal disorder
--proteinuria .0.5g/24 h or 3+, persistently
or cellular casts
- Neurological disorder
-- seizures
or psychosis (having excluded other causes e.g. drugs)
- Haematological disorder
-- haemolytic anaemia
or leucopenia of $< 4x10^9/l$ on two or more occasions
or lymphopenia of $< 1.5x10^9/l$ on two or more occasions
or thrombocytopenia$<100x10^9/l$
- Immunological disorders
-- positive LE cell
or raised anti-native DNA antibody binding
or false positive serological tests for syphilis, present for atleast six months
- Antinuclear antibody in raised titre

Table 1.1: Criteria for the diagnosis of SLE: A person shall be said to have SLE if four or more of the eleven criteria are present serially or simultaneously, during any interval of observation (Tan *et al.*, 1982).

SLE is a disease that affects predominantly women (female to male ratio- 9:1) of childbearing age (peak age of onset is between 20 and 40) with an incidence of approximately 1:4000 in Caucasians. The incidence of SLE displays wide ethnic variations. For example, in the Birmingham area (circa 1995) the prevalence of SLE in females was 20.6:10,000 in Afro-Caribbeans, 9.1:10,000 in Asians and 3.6:10,000 in Caucasians (Johnston *et al.*, 1995). The equivalent ratios for males were 0.93:10,000, 2.6:10,000 and 3.4:10,000 respectively. Similar ratios have been described by other groups who sampled patient populations in the USA and Europe (Fessel, 1974; Hochberg and Petri, 1993). Studies in twins suggest a concordance rate of between 30-70% for monozygotic twins compared to 3-15% for dizygotic twins. The fact that the concordance rate for monozygotic twins is not 100%, suggests that environmental factors have a role to play in addition to genetic factors in disease development (Isenberg, 1993; Isenberg and Horsfall, 1998).

1.1.1: Genetic Factors.

The genetic basis of lupus-like disease is remarkably complex, involving contributions from multiple genes in addition to the Class II MHC genes (Kotzin, 1996)

Multiple ethnic populations have been analysed for SLE susceptibility genes and three principles have emerged:-

a) For most ethnic populations, many susceptibility genes differ from those of other populations.

b) There are some single genes that predispose to disease across various ethnic groups.

c) Genetic predisposition is linked to auto antibody repertoires and clinical subsets of disease (Wakeland *et al.*, 2001).

The best-defined genes linked to SLE in humans are located on Chromosome 6 in the region that encodes the HLA genes. For example: -

HLA alleles A1, B8, DR3 – associated with lupus in Caucasians.

HLA DR2 – associated with lupus in Japanese

Some non- HLA genes and inherited complement deficiencies also predispose to lupus, as does IgA deficiency (Isenberg and Horsfall, 1998). Deficiencies in the early classical pathway complement components C1q, C2 and C4 are strongly associated with the

development of a lupus like disease (Haq et al., 2002). Immunoglobulin G (IgG) receptors on mononuclear cells clear IgG and IgG containing immune complexes from the circulation. In Afro-American patients, associations have been found between SLE and the presence of low-affinity IgG receptors (Haq et al., 2002).

A list of modified genes that have been reported to mediate some component of systemic autoimmunity in human SLE and murine models is provided in table 1.2. As seen in the table these genes can be organized into broadly defined pathways impacting immune function relevant to systemic autoimmunity. A hypothetical model of the roles the various genetic pathways play in the initiation of SLE pathogenesis has been proposed in a recent review by Wakeland et al., and outlined in figure 1.1. The development of SLE can be viewed as involving interactions between the genes of the three pathways namely, loss of tolerance to nuclear antigens, dysregulation of the immune system and end organ targeting (Wakeland *et al.*, 2001).

Proposed Mechanism	Murine SLE	Human SLE
Antigen/Immune complex clearance	C1q knockout	C1q
	C2, C3 knockout	C2, C3, C4
	SAP knockout	Mannose binding protein
	DNAase I knockout	$Fc_{\gamma}RIIA$
	Serum IgM knockout	$Fc_{\gamma}RIIIA$
	Fc_{γ} common chain knockout	DNAase I
	Mer Knockout	T cell receptor ζ chain
Lymphoid signalling	SHP-1 knockout	TNF-α
	Lyn Knockout	IL-10
	Lyn/Fyn double knockout	
	CD22 knockout	
	BlyS transgenic	
	PD1 knockout	
	IL-2 knockout	
	CD45 E613R point mutation	
	G2A knockout	
	IFN-γ transgenic	
Apoptosis	Fas knockout	
	Fas-L knockout	
	Bcl-2 transgenic	
	Pten heterozygous deficiency	
	P21 cyclin dependent kinase knockout	
Epitope modification	α-Mannoside II knockout	

Table 1.2: Candidate genes and pathways implicated in SLE.
(Wakeland *et al.*, 2001)

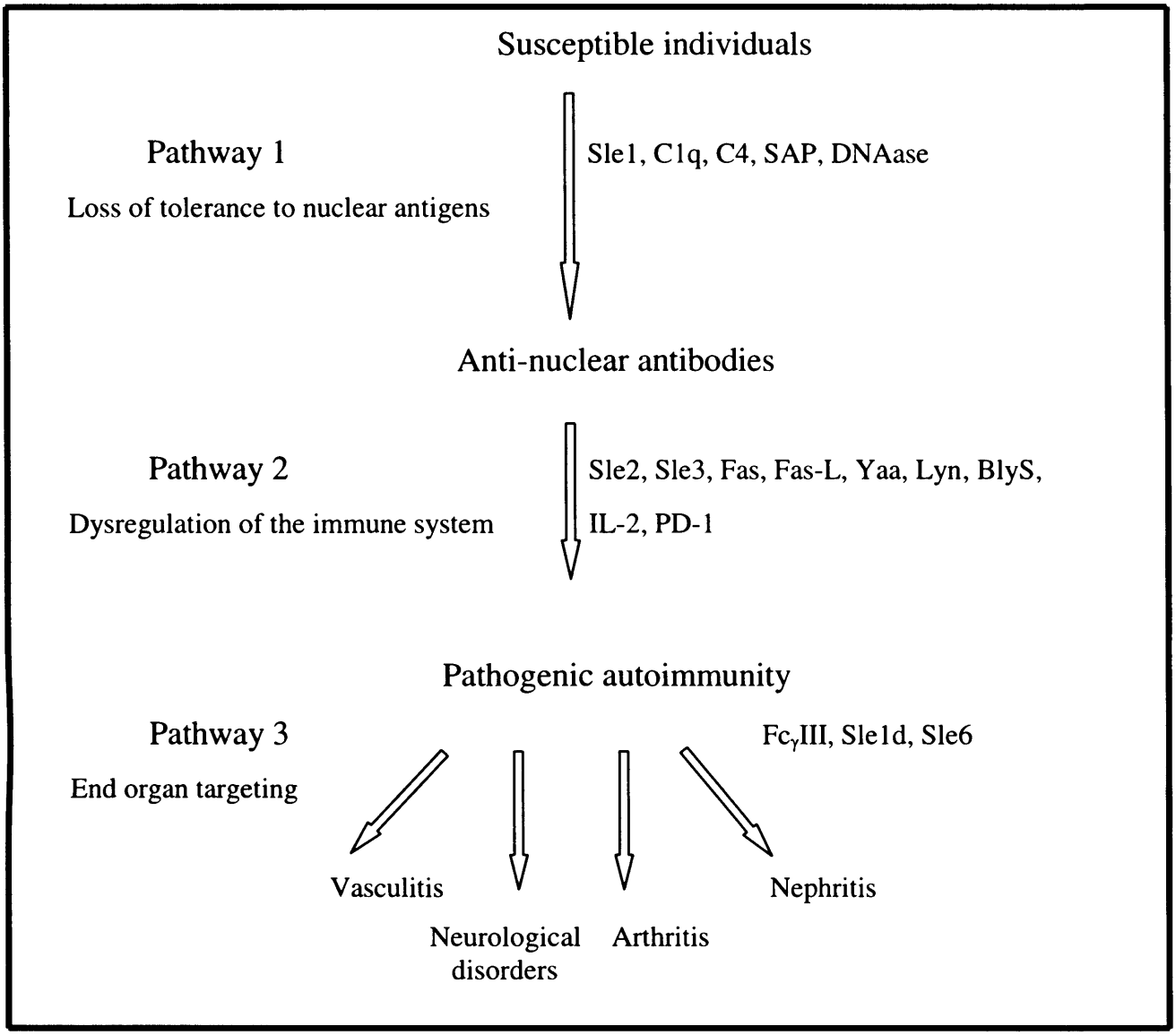


Figure 1.1: Hypothetical pathway proposed by Wakeland et al., illustrating the manner in which individual susceptibility genes interact to potentiate severe autoimmunity.

Sle: Systemic lupus erythematosus susceptibility foci.
C: complement protein.
SAP: Serum amyloid P component.
Yaa: Y-linked autoimmune accelerator.
Lyn: Src family tyrosine kinase.
BlyS: B lymphocyte stimulator.
PD-1: Programmed death 1 receptor.
IL-2: Interleukin 2.
Fc: Receptor for Fc region of immunoglobulin.

1.1.2: Environmental factors.

Environmental influences on the expression of disease manifestations are clearly seen in SLE and those that may play a role are summarised in table 1.3. Of these factors the two most influential are UV light and sex hormones. It has been reported that up to 70% of SLE patients have disease flared by exposure to UV light (Kelley, 1990). A possible explanation for this is that UV light increases thymine dimerisation, which renders the DNA more immunogenic. More recently the ability of UV light to trigger apoptosis has been highlighted (discussed in section 1.9.3).

The predisposition of the disease to affecting women of child bearing age is not well understood. Estrogens are believed to play a role in the etiology of both human and murine SLE (Lahita, 1999). Sex hormones have been shown to augment CD8 and CD4 lymphocyte-mediated cytolysis, to enhance production of IFN-γ and IL-10 by Th1 and Th2 cells, respectively (Kammer *et al.,* 1998). Rider et al, have reported that T cells from patients with SLE, but not normal or disease control cells, responded to estrogen by significantly increasing the amounts of calcineurin transcripts and calcineurin phosphatase (CN) activities (Rider *et al.,*1998). Calcineurin is a pivotal phosphatase in the biochemical cascade that conveys a signal from the T cell antigen receptor to the nucleus. The hypothesis proposed is that altered calcineurin regulation is a component of a primary T cell disorder encoded by multiple abnormal genes, manifested by T cell dysfunction, and resulting in increased autoimmunity (Kammer *et al.,* 1998). Recent research also suggests that estrogen acting through the estrogen receptor, enhances T cell activation in women with lupus resulting in amplified T-B cell interactions, B cell activation and autoantibody production (Rider and Abdou, 2001). Studies in mice also show that estrogen administration activates extrathymic T cell differentiation in the liver (Okuyama *et al.,* 1992). The resulting T cells have unique characteristics such as autoreactivity and might in part, explain the female predominance of autoimmune diseases (Okuyama *et al.*, 1992).

Drug-induced lupus was first recognised almost 50 years ago in association with hydralazine therapy, and numerous medications have been implicated in the *de novo* induction of rheumatological symptoms (Rubin, 1999). Drug-induced lupus has some features common with SLE and characteristically develops in people who have no history of systemic rheumatic disease. Medications currently in use and associated with at least several reports of lupus like syndrome are listed in table1.3. The highest risk drugs are procainamide and hydralazine

(Rubin, 1999, Ioannou *et al.*, 2002). *In vivo* metabolites of the drugs rather than ingested medication are thought to be responsible for the initiation of autoimmunity. The proposed mechanisms for autoantibody induction by lupus-inducing drugs include-

- Drug-altered B cell and/or T cell autoepitope,
- Drug directly activates B and /or T lymphocytes,
- Drug-specific T cells activate autoreactive B cells presenting drugs, and
- Drug prevents establishment of immune self-tolerance (Rubin, 1999).

Viral infections may be one of the factors that induce the production of autoreactive antibodies in patients with SLE, although the involved mechanisms are still incompletely understood. One proposed mechanism for virus-induced production of autoantibodies is molecular mimicry (Marilyn *et al.*, 2003). Another mechanism derives from studies with the human polyomavirus BK. In these studies, *in vivo* binding of the polyomaviruses large T-antigen to chromatin of infected cells may render chromatin immunogenic. The large T-antigen–chromatin complex may thus function as a hapten-carrier model with subsequent production of anti-chromatin antibodies, including anti-dsDNA and anti-histone antibodies (Marilyn *et al.*, 2003).

Definite
Ultraviolet B light Sex hormones In humans , female:male ratio is 9:1 between menarche and menopause, 3:1 in young and old
Possible Dietary factors Alfalfa and related sprout foods (L-Canavanine) High-calorie diets High intake of saturated fats Infectious agents Retroviruses Bacterial Lipopolysaccharides
Exposure to certain drugs: Hydralazine Procainamide Isoniazid Hydantoins Chlorpromazine Methyldopa D- Pencillamine Interferon-α

Table 1.3: Environmental factors that may play a role in SLE (Kelley, 1990)

1.2: Clinical assessment of SLE.

SLE is a complex multi-system disease. This complexity makes the disease difficult to monitor. In particular, there are problems in quantifying disease activity in the various organs/systems, and in differentiating potentially reversible organ dysfunction (due to active disease) from irreversible organ damage (Hay *et al.*, 1993). More than 60 scales for measuring clinical disease activity in patients with SLE have been developed and used in a variety of studies but few have been validated ot shown to be reliable (Hay *et al.*, 1993). Some of the most frequently used, validated global score systems are: the European Consensus lupus activity measure (ECLAM), systemic lupus erythematosus disease activity index (SLEDAI), systemic lupus activation measure (SLAM), and lupus activity index (LAI). In contrast the

British Isles Lupus Assessment group (BILAG) index provides an 'at a glance' view of activity in eight organs/systems. It is based on the 'physicians intention to treat' principle.

A number of studies of the SLEDAI, SLAM and BILAG disease activity indices have shown that the three indices are comparable and reproducible for evaluating disease in SLE (Gladman *et al.*, 1992; Vitali *et al.*, 1992). Most recently, when all these scores were used to assess a cohort of 19 patients, who were assessed fortnightly for 40 weeks, there was a statistically significant correlation between the results obtained from each score. During the study, 16 of the patients experienced a 96% or greater change in their disease activity as measured by the BILAG score (Ward *et al.*, 2000).The BILAG score was used to assess each patient included in this thesis (Hay *et al.*, 1993). The BILAG index is a computerised index for measuring clinical disease activity in SLE. The index allocates alphabetic scores (A-E) to each of the eight organs/systems (general, muco-cutaneous, neurological, musculoskeletal, cardiorespiratory, vasculitis and thrombosis, renal, haematological). The most active state is given an A grade while the complete absence of disease activity during the course of the disease gets an E. These individual organ grades were converted into a global score, with grade A=9, B=3, C=1 and D&E =0. A patient with a global score of more than or equal to 6 is regarded as having active disease and 0-5 inactive disease.

1.2.1: Clinical feature of SLE.

SLE has the potential to involve multiple organ systems directly, and its clinical manifestations are extremely diverse and variable. Musculoskeletal and dermatological involvement are the most common manifestations of lupus, with more than 90% of the patients having one or both systems affected (Haq *et al.*, 2002).

Arthralgia (90%), myalgia (50%), and tenosynovitis (20%) are common features in the musculoskeletal system. Avascular necrosis of the bone causes major morbidity in SLE and is associated with corticosteroid use. Osteoporosis is another major complication of SLE. It may result from lack of vitamin D caused by sun avoidance, menstrual cycle dysfunction from disease or treatment, corticosteroid use or lack of exercise (Haq *et al.*, 2002).

The heart and lungs are often involved – shortness of breath, pleurisy and pleural effusions are seen in about half of patients (Kelley, 1990). Pulmonary hypertension in SLE may result from multiple factors such as vasculitis, thrombosis and pulmonary artery vasoconstriction (Haq *et al.*, 2002). The prognosis is relatively poor. Thromboembolic disease

is associated with the presence of lupus anticoagulant and antiphospholipid antibodies and patients may need lifelong anticoagulation (Haq *et al.*, 2002).

Gastrointestinal system symptoms are common in SLE. Anorexia, nausea and vomiting occur in up to half of the patients (Haq *et al.*, 2002). Sjögren's syndrome may coexist with SLE and can cause gingivitis, accelerated dental caries and oral infections such as candidiasis (Haq *et al.*, 2002). Patients with SLE are at risk of peptic ulcer disease from the use of non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids (Haq *et al.*, 2002).

Until recently, renal disease was the major cause of death in SLE, and 30% of patients develop significant renal disease (Haq *et al.*, 2002). Symptoms can be minimal until substantial kidney damage has occurred (Haq *et al.*, 2002). The immunosuppressive therapies used to treat the involvement of the kidneys add greatly to the morbidity and mortality of SLE patients (Zimmerman *et al.*, 2001).

Skin involvement is another common occurrence and up to 40% of patients have characteristic butterfly rash- essentially a photosensitive vasculitis over the malar bones and bridge of the nose. Patients also show maculopapular discoid lesions and vasculitis lesions on fingers and toes (Haq *et al.*, 2002). The commonest causes of death from SLE are infection, malignancy or atherosclerosis (Kelley, 1990).

1.2.2: Immunopathology.

The result of interactions between susceptibility genes and triggering environmental factors is the development of an abnormal immune response characteristic of SLE. The primary pathogenic components responsible for causing SLE are elusive, because the primary immunogens that trigger the auto-immune response in the disease have not been clearly identified (Datta, 1998).

The common denominator among SLE patients is Immunoglobulin G (IgG) autoantibody production, and in particular the elevated serum levels of antibodies to nuclear constituents (i.e., anti-nuclear antibodies or ANAs)(Kotzin, 1996). Raised dsDNA antibody titres were found in 60% of the patients in a study of a cohort of 300 patients by Isenberg et al (Haq *et al.*, 2002). dsDNA antibodies are closely related to renal disease and an increase in titre, particularly if it is associated with decreasing C3 levels, alerts the clinician to a possible flare of disease (Haq *et al.*, 2002). These pathogenic autoantibodies are an effect of hyper

activity of auto-reactive B and T cells and the failure of the immunoregulatory circuits to control their activity (Kotzin, 1996).

1.2.3: Treatment of patients with SLE.

Successful therapy of patients with SLE depends upon targeting both the symptoms and the underlying infection. Both pharmacological and non-pharmocological therapies are used to treat patients with SLE (Ioannou *et al.*, 2002). The pharmacological management of patients with SLE presently revolves around four main classes of drugs, often in combination. These are:

- Non-steroidal anti-inflammatory drugs (NSAIDs).
- Anti-malarials.
- Corticosteroids.
- Cytotoxic drugs.

Patients with mild lupus are generally treated with a combination of NSAIDs and antimalarial drugs such as a hydroxychloroquine (Plaquenil) (Ioannou *et al.*, 2002). Low doses of corticosteroids are used when NSAIDs and antimalarials have failed to control symptoms of arthralgia/arthritis or rash sufficiently (Ioannou *et al.*, 2002). Treatment of patients with major organ involvement involves broad spectrum immunosuppression with cytotoxic agents (Zimmerman *et al.*, 2001). The drugs most frequently employed are azathioprine or cyclophosphamide together with corticosteroids, often in high doses. The use of cyclophosphamide, however, is limited by its side effect profile of profound nausea, alopecia, infertility especially in patients over the age of 30 and bone marrow suppression (Ioannou *et al.*, 2002). The Bloomsbury rheumatology clinic, attended by patients studied in this thesis were treated with oral prednisolone, with azathioprine in mild to moderately active lupus, reserving pulsed cyclophosphamide (intravenous pulses of cyclophosphamide) for severe renal involvement (Ioannou *et al.*, 2002).

Other treatments regimens used include plasmapheresis, dietary therapy, intravenous high dose gammaglobulins and cyclosporin therapy (Ioannou *et al.*, 2002). Plasmapheresis or plasma exchange was thought to benefit the patient by the removal of autoreactive antibodies, antigens, and immune complexes from the circulation (Zimmerman *et al.*, 2001). No

conclusive advantage of this form of treatment has emerged from clinical studies, and limitations in its use include a "rebound" synthesis of autoantibodies, technical difficulties requiring central venous access with associated complications, patient discomfort and being an expensive form of therapy (Zimmerman *et al.*, 2001, Ioannou *et al.*, 2002). Dietary therapy in the form of calorific and in particular fat restriction is beneficial to patients, as is total zinc reduction (Ioannou *et al.*, 2002). Studies have also shown the beneficial effects of a diet low in saturated fat combined with fish oil supplementation over a six-month period (Walton *et al.*, 1991). Calorie restriction, however, has no place in the patient with severely active lupus with major organ damage, as these patients tend to be acutely unwell and hence in a hypercatabolic state (Ioannou *et al.*, 2002). Intravenous immunoglobulin therapy has an established role in lupus patients with severe thrombocytopenia or immune neutropenia (Ioannou *et al.*, 2002). The intravenous administration of human immunoglobulin (Ig) could ameliorate symptoms by solubilizing immune complexes deposited in the kidney or by blocking Fc receptors on immune effector cells or mesangial cells of the kidney glomerulus (Zimmerman *et al.*, 2001). Recent evidence has also demonstrated that cyclosporin (CsA) in low doses (2.5-5mg/kg/day) may provide reasonable disease control and offer the opportunity for reduction in steroid over long term follow up (Ioannou *et al.*, 2002). Cyclosporin selectively inhibits T-cell function by blocking transcription of specific cytokines such as IL-2 and IFN-γ (Marilyn et al., 2002). The primary concern about the use of cyclosporin has been the potential for long-term nephrotoxicity, although other reversible side effects include hypertension, gingival hyperplasia and hypertrichosis (Zimmerman *et al.*, 2001).

Greater understanding of the immunopathogenic dysregulation characterising lupus in recent years, has enabled the development of targeted therapy to interfere with this dysregulation at various levels. Reagents near or at clinical trial phase include biological treatments developed to modulate or inhibit T cell activation (CTLA-4Ig, anti-CD40 ligand, anti-B7 monoclonal antibodies), T and B cell collaboration, anti-dsDNA antibody production (anti-CD20 mouse/human chimeric antibody monoclonal antibody), deposition of anti-dsDNA antibody complexes (DNase), complement activation/deposition (anti-complement 5 monoclonal antibody), and cytokine activation (anti-IL-10 antibody, recombinant IL-1) (Ioannou *et al.*, 2002, Marilyn et al., 2002).

1.3: Autoantibody production and disease manifestation.

The principle targets for the autoantibodies produced in SLE include protein-nucleic acid complexes, notably ds and ss DNA, histones and nucleosomes, the U1 and Sm small nuclear ribonucleoprotein(snRNP) particles and the Ro/SSA and La/SSB RNP complexes(Tan, 1989). Autoantibodies to phospholipids (complexed to β2 glycoprotein 1) are also relatively frequent and associated with thrombotic complications. A separate group of autoantibodies in SLE are directed to cell surface molecules, causing haemolytic anaemia and platelet destruction (thrombocytopenia)(Kotzin, 1996). The mechanisms by which most autoantibodies in lupus may cause disease is unclear. Anti-nuclear antibodies may on occasion penetrate cellular membranes and cause disease by binding to their intracellular targets (Alarcon-Segovia *et al.*, 1979). More often it is believed that, the disease manifestations such as lupus nephritis, arthritis, and vasculitis may due to the formation of immune complexes.

Antibody	**% prevalence**
Anti-nuclear antibody	96
Anti-Ro	25
Anti-La	19
Anti-Sm	10
Anti-U1RNP	13
Anticardiolipin IgG	24
Anticardiolipin IgM	13
Anti-ds DNA	70

Table 1.4: Prevalence of autoantibodies in patients with SLE (Cervera *et al.*, 1993).

Anti-double stranded DNA (anti-ds DNA abs) antibodies are the most prevalent form of anti-nuclear antibody (ANA) in SLE patients (Table 1.4). Genetic studies indicate that anti-dsDNA antibodies derive from an antigen selective stimulus (Radic and Weigert, 1994). These antibodies are usually oligoclonal, somatically mutated, and their V regions contain structures that are selected by, and bind to, double stranded DNA (Rekvig *et al.*, 1997). In contrast to anti-single stranded DNA antibodies (anti-ssDNA abs), anti-ds DNA antibodies are very rarely found in any disease other than SLE (Koffler *et al.*, 1971; Isenberg and Collins, 1985). Since, anti-dsDNA antibodies are common in, and specific to, SLE they represent a good candidate as a pathogenic factor in SLE. Therefore, their structure and relationship with disease activity needs to be characterised. The association of anti-ds DNA antibodies as one of the pathogenic factors in SLE is well established although while some studies find a strong correlation, particularly with vasculitis and glomerulonephritis, others find a weaker link (Esdaile *et al.*, 1996; Petri *et al.*, 1991). The disparity in the results of the various studies may be due to:

1. the differences in the detection methods used,

2. missing the expansion in antibody levels as they are sequestered to target organs (e.g., kidney) or

3. that in a particular cohort of patients tested there is no association.

It is however, generally agreed that rising anti-ds DNA titres increase the risk of disease exacerbation in the following 8-10 weeks by a factor of 2-3 (Hahn, 1998).

1.3.1: Anti-DNA antibodies.

Normal individuals produce "natural" anti-DNA antibodies of IgM class in high frequency (Datta *et al.*, 1986). In drug-induced lupus or patients with macroglobulinaemia, high levels of anti-DNA antibodies usually binding to ssDNA rather than dsDNA are produced with no disease manifestations as in SLE (Lahita *et al.*, 1979; Pisetsky *et al.*, 1990). Anti-histone antibodies are also a common feature of drug-induced lupus (Rubin, 1999).

In contrast to the natural (IgM) antibodies, pathogenic (e.g., nephritogenic) anti-DNA antibodies are of the IgG class (Theofilopoulos *et al.*, 1989). These IgG antibodies are cationic as described in murine models and accompany the onset of murine lupus nephritis (Furukawa, 1997; Datta *et al.*, 1987). The cationic anti-DNA antibodies and or cationic immune complexes

may form persistent immune deposits in glomeruli when injected into normal mice whereas the anionic (or neutral) antibodies and immune complexes do not persist (Shivakumar *et al.*, 1989). Thus, deposition of immune complexes consisting of cationic anti-DNA autoantibodies may initiate the renal lesions of lupus nephritis, which may then be followed by the deposition of anionic or neutral autoantibodies (Gavalchin and Datta, 1987). The nephritogenic potential of cationic anti-DNA antibodies or cationic immune complexes probably arises from their affinity for the glomerular anionic sites that are constituted by negatively charged proteoglycans (Datta *et al.*, 1987). Deposition of antibodies on the glomerular basement membrane results in recruitment of complement components and engagement of Fc receptors either on renal cells or on circulating monocytes (Schiffer *et al.*, 2002). This deposition precipitates an inflammatory cascade in which upregulation of adhesion molecules and renal inflammatory chemokines attracts more lymphoid cells upregulation of lymphoid derived chemokines enhances influx of cells into the kidney. Release of cytokines and other inflammatory mediators results in tissue damage that finally leads to irreversible cell death (Schiffer *et al.*, 2002).

Isolated B cells from both active and inactive lupus patients spontaneously produced markedly increased levels of total polyclonal IgG as compared to normal subjects but only B cells from active patients could produce pathogenic (cationic) anti-DNA autoantibodies with the help of autologous T helper cells (Shivakumar *et al.*, 1989). These cationic anti-DNA antibodies produced by B cells (cultured *in-vitro*) isolated from patients with SLE with active disease (lupus nephritis) are found to be strikingly restricted in spectrotype. This observation indicates that the Th cells (T helper cells) help an oligoclonal population of B cells that are committed to produce this pathogenic variety of autoantibodies in patients with active disease. Studies in murine models of lupus nephritis have also shown that the pathogenic (cationic) variety of anti-DNA antibodies share a common antigenic specificity pattern and cross-reactive idiotype. These antibodies are distinct from nonpathogenic (natural) autoantibodies, indicating that they are produced by a select and oligoclonal population of B cells (Gavalchin and Datta, 1987; Hahn and Ebling, 1984). Thus, it has been concluded that in humans and mice with active SLE, complex cellular mechanisms specific for the induction of pathogenic anti-DNA antibodies are operative. This discreet immunoregulatory defect could involve select subsets of T (helper) cells and B cells (Shivakumar *et al.*, 1989)

1.4: Cellular abnormalities in SLE.

Studies show that the cellular immune disorder of SLE is complex including multicellular dysfunction of all mononuclear leukocytes, including monocytes, macrophages, natural killer cells, B lymphocytes and T lymphocytes (Dayal and Kammer, 1996). Some of the major cellular and cytokine abnormalities in SLE are briefly summarised in table 1.5. In general, T and B cells manifest evidence of intense *in vivo* activation, but are hypo-responsive to exogenous antigens *in vitro (*Cohen, *1993).*

Cells	Defect
Monocytes/Macrophages	Decreased TNF-α production
Lymphocytes	
B cells	Increased numbers of activated B cells
	Hypergammaglobulinaemia, with a wide range of autoantibodies
	Increased CD25 expression
	Decreased CR1 expression
	Increased cytoplasmic and surface expression of hsp90
T cells	Decreased numbers of $CD4^+$ $CD45RA^+$cells
	Increased numbers of activated T cells ($HLADR^+$)
	Increased numbers of αβ TCR^+ DN ($CD3^+CD4^-$ $CD8^-$) T cells
	Increased and prolonged expression of CD40L on T cells
	Defective supression/regulation
	Impaired cytotoxicity

Table 1.5: Some major cellular abnormalities in SLE. Table adapted from Isenberg, 1993.

1.4.1: Role of B cells in SLE.

B cells play a pivotal role in SLE. They are the source of the pathogenic autoantibodies, some of which may be harmful, others may serve as potentially useful diagnostic markers (Cohen, 1993). Their role is not limited to secretion of autoantibodies and it seems unlikely that SLE is solely an immune complex mediated disease. Under certain conditions, B cells can activate memory T cells, and process and present self antigens to naïve T cells, implying the existence of an antibody-independent mechanism for tissue injury in systemic autoimmune diseases, such as lupus (Zouali, 2002).

It is evident that switching of both antibody isotype and idiotype may be involved in the immunopathology of lupus. IgG antibodies are more pathogenic than IgM and certain idiotypes (e.g. Id 16/16) have been found in the serum of the majority of lupus patients and in many renal biopsies (Haq *et al,* 2002).

There is often a marked increase in the numbers of plasma cells in the peripheral blood and in the numbers of B cells at all stages of activation (Kelley, 1990). Studies of twins and family members have shown that such increased numbers can precede a clinical disease (Kelley, 1990).

In mice, polyclonal activation of B cells precedes the accumulation of high avidity single autoantigen specific B cell repertoire that characterises well-developed disease (Kelley, 1990). This suggests that B cells in mice are subject to both increased polyclonal and specific activation (Dziarski, 1988). Individual autoantibody repertoires vary from person to person- although all have ANA's, some have anti-dsDNA antibodies, others have anti-Ro antibodies etc (Kelley, 1990). Hence it is likely that many B cells are activated by specific autoantigens although the basis for this hyperactivity is unknown. However, it has been reported that SLE B cells are more easily activated and driven to mature by cytokine stimulation than B cells from normal individuals. For example, B cells from SLE patients are more easily driven to differentiate by IL-6 than are normal B cells (Kelley, 1990).

1.4.2: Role of T cells in SLE.

B cell hyperactivity and autoantibody production are considered to be central to the pathophysiology of SLE. T cells have been postulated to have a major role in driving the SLE associated B cell hyperactivity based on observations that:

- Much of the pathology in SLE is mediated by autoantibodies of the IgG class, a T cell dependent response (Cohen, 1993; Via *et al.*, 1993; Spronk *et al.*, 1996)

- *In vivo* manipulations in murine SLE which reduce or inhibit T cell numbers and /or function have been shown to improve disease parameters (Via *et al.*, 1993; Wang *et al.*,2002)

- There is an association of SLE with particular class II major histocompatability complex (MHC) alleles (Kotzin, 1996)

The specificities of the autoreactive T cells, however have not been well characterised and the nature of T cell help in SLE may differ from conventional responses. The emergence of these autoimmune T and B cells in SLE is probably determined by genetically programmed defects of the immune system. Environmental and other endogenous factors may play a secondary role in lupus as non-autoimmune subjects are also exposed to the same factors (Datta, 1998). The question remains whether the T cell dysfunction in SLE reflects an intrinsic disorder(s) and/or a faulty interaction between antigen presenting cell (APC) and T cell.

1.4.3: Primary T cell disorders in SLE.

Studies demonstrating both biochemical and functional abnormalities in T cells from patients with SLE have led to the hypothesis of a primary T cell disorder in SLE. This primary T cell disorder could result in altered regulation and/or expression of genes that impair T cell immune effector functions. Although substantial gaps in the knowledge of the primary T cell disorders remain, dysfunctional signalling has been identified in both the adenyl cyclase/cAMP/protein kinase A and phosphoinositide/Ca^{++}/protein kinase C pathways in murine and human lupus (Dayal and Kammer, 1996). CD3-mediated increases in free intracytoplasmic calcium occur specifically in lupus T cells and lines. This finding provides additional evidence that cell signalling events are defective in patients with lupus (Tsokos, 1996). The increased calcium responses in lupus T cells may account for the altered apoptosis,

altered PKA function or decreased IL-2 production reported in SLE (Tsokos, 1996). The presence of these defects in the T cell signal transduction system could significantly modify the T cell's capacity to express particular genes in response to certain stimuli resulting in altered gene expression. Altering gene expression of cytokines for example, could potentially lead to a shift in the predominant T cell cytokine pattern (e.g., Th0→ Th2 switch), hindering physiological T cell effector and regulatory function.

1.4.4: T cell –Antigen presenting cell (APC) interactions in SLE.

Defects in antigen presenting cell surface molecule expression and function have been reported in patients with SLE (Tsokos *et al.*, 1996). Abnormal APC function has been supported by studies evaluating the response of T cells from patients with SLE to the recall antigen influenza virus and alloantigens (Via *et al.*, 1993) . Studies showing that the need for APC delivered co-stimulation is bypassed by effectively cross-linking CD28 or CD26 T cell surface molecules also point to impaired APC function (Sfikakis *et al.*, 1994; Plana *et al.*, 1994). APCs from patients with SLE have also been found to be defective in their expression of the B7-1 (CD80) costimulatory molecule. Resting normal APCs do not express B7-1, but treatment with interferon gamma (IFN-γ) induces its expression. APCs from SLE patients failed to express surface B7-1 following stimulation with IFN-γ and this defective expression is thought to be responsible for the decreased response of lupus T cells to recall antigens (Tsokos *et al.*, 1996; Scheinecker *et al.*,).

1.5: Cytokines in SLE.

The development of an effective immune response involves lymphoid cells, inflammatory cells, and haematopoietic cells. The complex interactions among these cells are mediated by a group of secreted low-molecular weight proteins that are collectively called cytokines to denote their role in cell to cell communication.

Cytokines play an essential role in moulding the quality of immune response to foreign or self-antigens. These mediators have been classified according to their cellular source and effector functions, with the Th1 and Th2 families best illustrating this division of labour. The Th1 cytokines include IL-2 and interferon-γ (IFN-γ), which in general promote delayed-type hypersensitivity responses by induction of T cell proliferation and monocyte activation. The

Th2 cytokines include IL-4, IL-5, IL-6 and IL-10 and promote humoral immune responses (Singh *et al.*, 1999; Van Eden *et al.*,). T cells expressing cytokines of both patterns have been designated Th0. Th0 cells represent a heterogeneous population of partially differentiated effector cells comprising multiple discrete subsets that can secrete Th1 and Th2 cytokines (Romagnani, 1997). Furthermore, T cells producing transforming growth factor-β (TGF-β), IL-4 and IL-10 have been described as Th3 cells, and are considered regulatory cells that can act to induce immune tolerance (Dean *et al.*, 2000)

Cytokines have been suggested to play an important role in the immune dysregulation observed in patients with SLE and murine lupus prone strains. The abundant data on the expression, activity, or response to different cytokines in patients or murine models have been controversial (Horwitz and Jacob, 1994; Singh *et al.*, 1999; Dean *et al.*, 2000).The analysis of cytokines in disease states, in general, has been complicated by variable results derived from studying serum levels, secretion into culture supernatants or *ex vivo* analysis of intracellular mRNA or proteins (Horwitz, Stohl,Gray, 1997). Cytokine abnormalities in patients with SLE were first documented in studies of IFN-α, the first cytokine to be described. IFN-α now has been shown by several groups to be elevated in sera from patients with SLE, although the cellular source and stimulus for the production of that cytokine are not yet defined (Hooks *et al.*, 1982). It is important to note that cytokine production is not only changed in patients with SLE when compared with autoimmune disease controls (RA) or healthy subjects, but also changes with different disease phenotypes. For example, IL-6 seems to be increased in the cerebrospinal fluid (CSF) of patients with central nervous system (CNS) involvement in SLE but not in patients with SLE who lack neurological symptoms (Dean *et al.*, 2000). It may be that as in other inflammatory diseases, the balance of cytokines is more important in determining disease phenotype or severity rather than determining disease susceptibility. Aberrant (increased or decreased) production of several cytokines including IL-1, TNF-α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IFN-γ and IFN-α have been reported in patients with SLE and murine models of lupus (Dean *et al.*, 2000).

1.5.1: The Th1/Th2 paradigm: role in SLE.

Th1 and Th2 T cells were first described by Mosmann et al., who discovered that T cells could be conveniently divided into two subsets, Th1 and Th2, with distinct arrays of cytokine secretion patterns and functions (Mosmann et al., 1996; Mosmann et al., 1986). A hypothesis was subsequently formulated according to which cell-mediated autoimmune diseases such as insulin-dependent diabetes mellitus are induced by Th1 cells and their cytokines and humoural-mediated autoimmune diseases are mediated by Th2 cells and their cytokines.

The respective contributions of Th1 and Th2 cytokines to the pathogenesis of SLE are still a matter of debate. Several Th2 cytokines, e.g., IL-6 and IL-10, are known to promote antibody formation by B cells, supporting the hypothesis that Th2 cells play an active role in the development of autoantibody-mediated autoimmune diseases such as SLE (Romagnani, 1997; Kirou and Crow, 1999; Horwitz and Jacob, 1994; Dean *et al.*, 2000). Further, treatment of MRL-lpr lupus prone mice with IL-4R or anti-IL-4 antibodies resulted in reduced mortality and disease (Nakajima *et al.*, 1997; Schorlemmer *et al.*, 1995). In addition, administration of IL-6 promoted and anti IL-6 inhibited disease in the MRL-lpr lupus prone mice (Ryffel *et al.*, 1994; Finck *et al.*, 1994). However, immune responses involving the Th1 phenotype have also been found in the both patients with SLE and in murine models of lupus (Theofilopoulos *et al.*, 2001 ; Kirou and Crow, 1999). For example, exacerbation of SLE by repeated injections of IFN-γ, and inhibition by anti-IFN-γ antibodies in (NZBxNZW) F_1 hybrid mice, suggest the involvement of Th-1 type cells in the spontaneous development and progression of SLE (Jacob *et al.*, 1987). Further support for a pathogenic role for the Th1 cytokine IFN-γ was the demonstration that MRL/lpr mice lacking IFN-γ or IFN-γ receptor had no glomerulonephritis (Schwarting *et al.*, 1998). A kinetic study of disease progression in various strains of MRL mice correlated disease with increased expression of IFN-γ, a relative decrease in production of IL-4, and IgG2a and IgG3, compared with IgG1, autoantibody levels. (Takahashi *et al.*, 1996; Reininger *et al.*, 1996). Taken together these findings have led to the suggestion that Th1 cytokines may initiate disease, followed by production of autoantibodies and secretion of proapoptotic and proinflammatory cytokines, mainly derived from monocytes. Th2 cytokines, while potentially protective, may be inadequate in lupus mice (Santiago *et al.*, 1997).

Recent studies in patients with SLE also indicate a role for monocytes in the generation of potentially pathogenic cytokines such as IL-6 and IL-10, known to enhance B cell growth and differentiation and contribute to autoantibody formation (Linker-Israeli *et al.*, 1991; Linker-Israeli *et al.*, 1999). Regulation of IL-6 expression is achieved through positive and negative signals. Studies have shown that some cytokines particularly IL-1β and TNF-α stimulate IL-6 while IL-4, IL-10 and TGF-β inhibit IL-6 secretion by PBMC from patients with SLE *in vitro (*Linker-Israeli *et al., 1999).*

Therefore it is likely that both Th1 and Th2 cytokines can modify a given autoimmune disease such as SLE depending on various factors such as stage of disease, local versus systemic expression and genetic background and without strict adherence to the Th1 versus Th2 dualism.

1.6: Nucleosomes as autoantigens in SLE

The cardinal feature of systemic lupus erythematosus is the formation of anti-nuclear antibodies notably those directed against DNA. Some of these antibodies react with the native (double-stranded) B helical form of DNA, while others only react with denatured (single-stranded) DNA(ssDNA). Most healthy subjects have a natural autoantibody repertoire including anti-DNA antibodies (Pisetsky *et al.*, 1990). The anti-DNA antibodies in healthy subjects show only a weak affinity for DNA and wide cross-reactivity for other antigens, and are mostly of the IgM isotype. In contrast, anti-DNA antibodies involved in the pathogenesis of SLE are of the IgG isotype and display high affinity for DNA. These antibodies bear all the characteristics of an antigen driven, T cell dependent immune response (Desai-Mehta *et al.*, 1995).

Although for a long time dsDNA has been believed to be a major autoantigen in SLE, dsDNA does not occur as such *in vivo* and also has poor immunogenicity in animal models. DNA outside the cell is generally present in the form of nucleosomes generated by apoptosis. Nucleosomes appear to be the particles that provide DNA *in vivo* and possibly become more immunogenic due to apoptosis induced modifications (Berden *et al.*, 1999; Bruns *et al.*, 2000).

The role of nucleosome as an autoantigen in SLE is further strengthened by the presence of nucleosome specific T helper cells and the high prevalence of nucleosome specific antibodies in patients with SLE (Bruns *et al.*, 2000)

T cell autoimmunity to nucleosomes may be a latent property of the immune system, but the spontaneous expansion of nucleosome-reactive T cells is a lupus specific event (Lu *et al.*, 1999; Bruns *et al.*, 2000; Andreassen *et al.*, 2002). A recent hypothesis by Datta et al, proposes that a sustained hyper-expression of CD40 ligand by lupus T cells, instigates tolerogenic dendritic cells or resting B cells to present nucleosomes in an immunogenic fashion (Kaliyaperumal *et al.*, 2002). Only certain epitopes in nucleosomes have been found to be dominantly recognised by the autoimmune T and B cells of SLE patients. By testing overlapping synthetic peptides spanning the core histones in nucleosomes, autoepitopes for murine and human lupus were identified. Autoimmune T cells from lupus prone mice, as well as patients with SLE were shown to be spontaneously primed to these disease relevant epitopes (Lu *et al.*, 1999). Anti-dsDNA and anti-ssDNA antibodies were found to be cross reactive with nucleosome antigens and conversely, antinucleosome antibodies with DNA. This cross reactivity of the antibodies is commonly explained by epitope spreading (Monneaux *et al.*, 2001).

The pathogenic impact of antinucleosome antibodies in human SLE is highlighted by a strong correlation with a more severe disease course, namely nephritis. Nucleosomes have been shown to bind to the glomerular basement membrane (GBM), via their histone proteins. Glomerulonephritis is then caused by the formation of nucleosome-anti-nucleosome antibody immune complexes (Van Bruggen *et al.*, 1997).

Nucleosomes *in vivo* are generated by the process of apoptosis. There is growing evidence that in systemic lupus erythematosus, apoptosis is disturbed, leading to the release of nucleosomes. Moreover, the immunogenicity of these autoantigens might be enhanced by apoptosis induced modifications. Impaired clearance of apoptotic cells might be another factor contributing to the abundance of autoantigens (e.g., nucleosomes) in SLE (Bruns *et al.*, 2000).

1.7: Apoptosis

Apoptosis from the Greek meaning "falling off" refers to the morphological appearance of cells dying by default (e.g. in embryogenesis) or following active initiation of a programmed pathway by a variety of soluble and surface signals. The importance of programmed (apoptotic) versus toxic (necrotic) cell death is that the former results in the ordered fragmentation of the cell. Cell fragments are degraded and phagocytosed by neighbouring cells or professional phagocytes, which are not activated and therefore do not cause inflammation.

Multiple signalling and effector pathways that mediate active responses to external growth, survival, or death factors control apoptosis. The defining characteristic of apoptosis is a complete change in cellular morphology. Cells undergo shrinkage, chromatin margination, membrane blebbing, nuclear condensation and then segmentation, and division into apoptotic bodies which may be phagocytosed, often within several hours (Cohen, 1993). Apoptosis or programmed cell death is distinct from accidental cell death (necrosis). Numerous morphological and biochemical differences that distinguish apoptotic from necrotic cell death are summarized in table 1.6.

Apoptosis	Necrosis
Morphologic criteria	
Deletion of single cells	Death of cell groups
Membrane blebbing, but no loss of integrity	Loss of membrane integrity
Cells shrink, ultimately forming apoptotic bodies	Cells swell and lyse
No inflammatory response	Significant inflammatory response
Phagocytosis by adjacent normal cells, macrophages	Phagocytosis by macrophages and some
Lysosomes intact	Lysosome leakage
Compaction of chromatin into dense masses	Clumpy, ill-defined aggregation uniformly of chromatin
Biochemical Criteria	
Induced by physiological stimuli disturbances	Evoked by nonphysiological disturbances
Tightly regulated process with synthetic steps	Loss of regulation of and ion activation homeostasis
Requires energy	No energy requirements
Requires macromolecular synthesis	No requirements for protein or nucleic acid synthesis
De novo gene transcription	No new gene transcription
Non-random oligonucleosomal length DNA	Random digestion of fragmentation of DNA

Table 1.6: Morphological and biochemical differences between apoptotic and necrotic cell death. Table adapted from (Elkon, 1994; Seki *et al.*, 1998).

1.7.1: Regulation of apoptosis

Over the past decade, our understanding of apoptosis, or programmed cell death, has increased greatly, with the identification of some of the major components of the apoptotic program and the processes regulating their activation. Although apoptosis is an intrinsic process present in all cells, it can be regulated by extrinsic factors, including growth factors, cell surface receptors, cellular stress and hormones.

Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis, the cell suicide program critical for development, tissue homeostasis, and protection against pathogens. The *bcl-2* gene was originally identified at the chromosomal breakpoint of t(14;18) –bearing B-cell lymphomas. The Bcl-2 family of proteins can somehow register diverse forms of intracellular damage, gauge whether other cells have provided a positive or negative stimulus, and integrate these competing signals to determine the cell's fate. Members of the Bcl-2 protein family most similar to Bcl-2 promote cell survival by inhibiting adapters needed for the activation of proteases (caspases) that dismantle the cell. More distant relatives instead promote apoptosis, apparently through mechanisms that include diplacing adapters from the pro-survival proteins (Reed, 1997; Adams and Cory, 1998). Pro- and anti-apoptotic family members can heterodimerize and seemingly titrate one another's functions, suggesting that their relative concentration may act as a rheostat for the suicide program (Oltvai *et al.*, 1993). At least 15 Bcl-2 family members have been identified in mammalian cells and several others in viruses. A list of some of the better known members of the Bcl-2 family is given below in table 1.7.

Pro-apoptotic BCl-2 family members	Anti-apoptotic Bcl2 family members
Bax	Bcl-2
Bak	Bcl-x_L
Bok	Bcl-w
Bad	Mcl-1
Bim	
Bid	

Table 1.7: The Bcl-2 family of pro- and anti- apoptotic proteins.

Elegant studies by Oltvai et al., have demonstrated that Bax counters the death repressor activity of Bcl-2 and proposed a model in which the inherent ratio of Bcl-2 to Bax determines the susceptibility to death following an apoptotic stimulus (Figure 1.2) (Oltvai *et al.*, 1993).

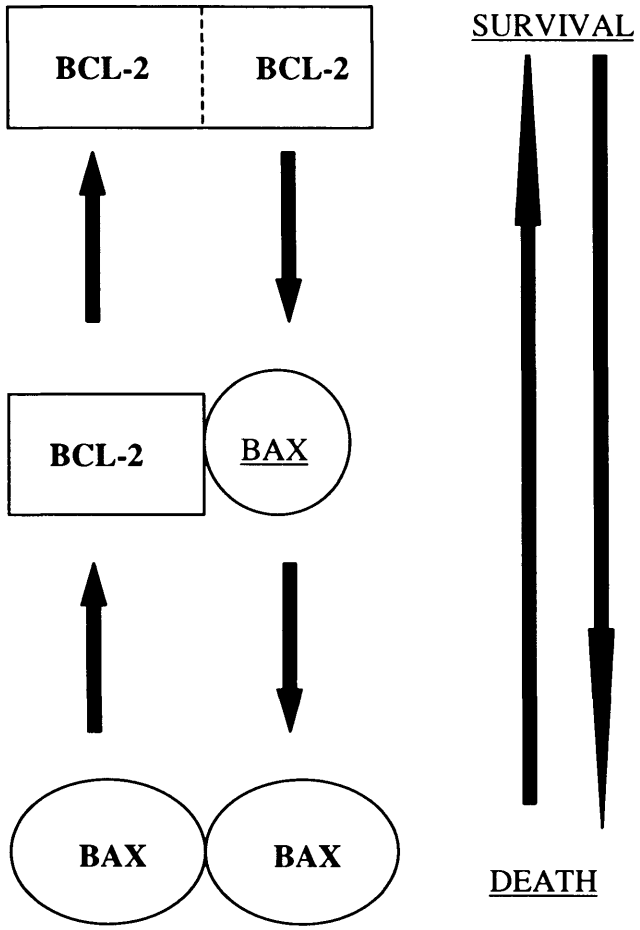


Figure 1.2: Model of interrelationship of Bcl-2 andBax and the regulation of programmed cell death (Oltvai *et al.*, 1993).

In 1993, Boise et al., reported the isolation of Bcl-x, a Bcl-2 related gene that could function as a Bcl-2-independent regulator of apoptosis. Alternative splicing of the Bcl-x mRNA was found to result in two distinct forms of the mRNA. The protein product of the larger mRNA, Bcl-x_L, was similar in size and predicted structure to Bcl-2. Surprisingly, the second mRNA species bcl-x_S, encodes a protein that inhibits the ability of Bcl-2 to enhance the survival of growth factor-deprived cells. Thus, the authours concluded that Bcl-x plays an important role in both positive and negative regulation of apoptosis (Boise *et al.*, 1993).

1.7.2: Apoptosis Dysregulation in SLE

Recent research shows that the genes with a distinct expression pattern in autoimmunity are not necessarily "immune response" genes, but are genes that encode proteins involved in apoptosis, cell cycle progression, cell differentiation, and cell migration. A large number of genes that encode proteins involved in multiple apoptosis pathways were found to be under expressed in PBMC's of patients with SLE, when compared to healthy controls(Maas *et al.*, 2002). These findings highlight the impact of defective apoptosis pathways on the pathogenesis of autoimmune disorders such as SLE.

Several molecules known to influence the induction of apoptosis have been studied in patients with SLE to evaluate their role in disease pathogenesis. The molecules studied in most detail are the Fas-FasL apoptotic pathway and the Bcl-2 family of genes.

The study of spontaneous and induced apoptosis in SLE lymphocytes has yielded conflicting results. Earlier studies indicated accelerated *in vitro* and *in vivo* apoptosis of lymphocytes from patients with SLE although, a more recent study reports lack of consistent abnormalities (Emlen W. *et al.*, 1994; Lorenz *et al.*, 1997; Perniok *et al.*, 1998; Caricchio and Cohen, 1999)

1.7.3: The Fas receptor in SLE

Fas/APO-1 (CD95) is a 48 kDa cell surface glycoprotein receptor belonging to the tumour necrosis factor(TNF)/ nerve growth factor (NGF) rceptor family(Nagata and Golstein, 1995). Cross-linking of Fas by Fas ligand (FasL), a 40 kDa membrane glycoprotein, triggers apoptotic cell death in many cell types.

Increased Fas expression has been documented on peripheral blood mononuclear cells (PBMC) of patients with SLE when compared to healthy control individuals. The increased Fas expression was observed in both B and T cells although expression was higher on $CD8^{+}$ve T cells (Mysler *et al.*, 1994; Lorenz *et al.*, 1997; Bijl *et al.*, 2001). Conversely, other reports suggest that most patients with SLE do not appear to have a defect in Fas expression or function (Ohsako *et al.*, 1994; Yang *et al.*, 1997; Caricchio and Cohen, 1999). However, levels of soluble Fas (sFas), a secreted variant with the potential to block Fas-L has been detected in the serum of 10-60% of patients with SLE, although a similar rise was also observed in patients with RA (Rose *et al.*, 1997; Jodo *et al.*, 1997). Serum levels of sFas are also reported

to correlate with percentages of activated B cells but not with that of activated T cells (Bijl *et al.*, 1998).

1.7.4: Fas Ligand in SLE

Fas-L induces apoptosis upon binding to Fas/Apo-1 (CD95) bearing target cells and plays an important role in activation induced cell death in T cells upon crosslinking of the T cell receptor (TCR) (Nagata *et al.*, 1995). Patients with active lupus showed increased Fas-L expression on PBMCs, especially in the activated T cell subset. The function and regulation of Fas-L in T cells from patients with SLE was found to be within normal parameters (Kovacs *et al.*, 1997; McNally *et al.*, 1997; Feng, 1997) . The increased expression of Fas-L on activated T cells of patients with SLE could account for the observation of increased apoptotic rates in some patients (Kovacs *et al.*, 1997).

1.8: bcl-2 family of genes in SLE

Many genes are involved in survival, proliferation and programmed cell death or apoptosis. Among them are the *bcl-2* gene family, p53, c-myc, the large families of TNF related receptors and ligands, as well as the caspase family.

bcl-2 the proto-oncogene contributing to cell survival has been studied extensively in the context of apoptosis in patients with SLE, often with conflicting findings. Expression of Bcl-2 protein on freshly isolated PBMC has been controversial, with some investigators showing increased Bcl-2 expression on T cells but not B cells and other groups showing unaltered or decreased Bcl-2 quantities in unfractioned PBMC from patients with SLE (Rose *et al.*, 1995; Chan *et al.*, 1997; Aringer *et al.*, 1994; Ohsako *et al.*, 1994; Graninger *et al.*, 1992 ; Graninger, 1992; Falcini *et al.*, 1999; Miret *et al.*, 1999). A further study found no detectable differences in Bcl-2 protein expression immediately after isolation of the cells, but significantly up-regulated levels in T lymphocytes from patients with SLE after three days of culture, in complete medium. The up-regulated Bcl-2 levels were accompanied by an increased rate of *in vitro* apoptosis in the T cells (Lorenz *et al.*, 1997). The same results were also observed with other autoimmune disease controls (Mixed connective tissue disease (MCTD), Wegner's granulomatosis, Takayasu arteritis, polyarteritis nodosa) and the authors

concluded that the increased *in vivo* activation levels of PBMC from autoimmune patients might explain these phenomenon (Lorenz *et al.*, 1997).

The study of other apoptosis related gene products such as Bcl-xL and Bax in patients with SLE, found no differences in levels of expression on PBMC when compared to healthy subjects (Lorenz *et al.*, 1997; Graninger *et al.*,). In bone marrow cells, however, decreased levels of Bax mRNA and protein have been reported both in patients with SLE and in lupus prone NZB/W mice. It has been suggested that this decreased expression of Bax in bone marrow might lead to decreased apoptosis in mature blood cells, resulting in autoreactive cells in the secondary lymphoid organs (Nazareth *et al.*, ; Alvarado-de *et al.*, 1998).

1.9: Impact of defective Apoptosis on SLE

It is now widely agreed that defective regulation of apoptosis (increased apoptosis or impaired clearance of apoptotic cell material) plays a role in the development of autoimmune diseases including SLE. As the autoantibody production in SLE is antigen driven, and involves T and B cell contributions, defective regulation of apoptosis in patients with SLE might impact disease pathogenesis in the following ways-

- Cause impaired tolerance resulting in the survival of autoreactive T and B lymphocytes
- Prolonged survival of activated autoreactive T and B lymphocytes
- Increased levels of apoptosis and/or defective clearance of apoptotic cells - the source of autoantigens such as nucleosomes.

1.9.1: Impaired Tolerance

Defects in the apoptotic pathway may increase the likelihood that lymphocytes avoid the normal processes used by the immune system to eliminate unwanted lymphocytes. Normally, autoreactive lymphocytes that survive the process of thymic selection and circulate in the periphery can respond to self antigen if presented by the correct MHC. This process is held in check by the combined actions of apoptosis and anergy and results in the state of peripheral tolerance. However, in patients with SLE, genes required both for apoptosis and induction of tolerance have been found to be under-expressed (Maas *et al.*, 2002). This may

permit lymphocytes to escape tolerance and adopt a pro-survival agenda that results in the formation of autoantibodies. The isolation of autoreactive T cell clones from murine lupus models and patients with SLE that support polyclonal autoantibody production by autologous B cells is consistent with this theory (Chen *et al.*, 1997; Takeno *et al.*, 1997; Shivakumar *et al.*, 1989).

1.9.2: Activation induced cell death in SLE

Activation of mature peripheral T cells through repeated cycles of antigen recognition and clonal proliferation leads to activation induced cell death by apoptosis (Nagata and Golstein, 1995). Activation induced cell death (AICD) plays an important role in the regulation of immune response by limiting the unwanted expansion of T cell clones reacting against foreign antigens beyond the course of infection, as well as the clones, which cross-react with self-antigens. Activated T cells from patients with SLE have been shown to be relatively resistant to a T cell receptor (TCR) mediated stimulus (Kovacs *et al.*, 1996). This defective antigen-mediated cell death could contribute to increased numbers of activated autoreactive cells in SLE patients (Kovacs *et al.*, 1996). Activated T cells from patients with SLE have also been shown to be resistant to apoptosis induced by human thymic stromal cells pointing to a defect in the apoptosis pathway (Budagyan *et al.*, 1998).

1.9.3: Clearance of Apoptotic cells in SLE

It has been speculated that autoantigens released from apoptozing cells may contribute to the aetiopathogenesis of SLE by both activation of autoreactive lymphocytes and the formation of immune complexes. This theory is supported by studies where modest transient antibody production has been documented in animals immunized with apoptotic cells (Mevorach, 1999). In other systems, T cell reactivity to transfected antigens contained within apoptotic cells *in vivo* and *in vitro* has been observed, and is enhanced when appropriate costimulatory molecules and cytokines are present (Rovere *et al.*, 2000; Rovere *et al.*, 1999). The autoantigens targeted in SLE have been found to be clustered in the surface blebs of apoptotic cells, where they are vulnerable to oxidative modifications. The revelation of previously cryptic antigens from apoptotic cells to the immune system, by these oxidative modifications may result in the autoimmune reaction of SLE (Casciola-Rosen *et al.*, 1994).

Vast numbers of apoptotic cells are generated through cell senescence, maturation, and turnover. Multiple macrophage scavenger receptors serve the important function of

recognising and promoting engulfment and removal of apoptotic cells (Fadok *et al.*, 2001). Several recent reports have emphasised that defective macrophage clearance of apoptotic debris may lead to autoimmunity. Delayed apoptotic cell clearance in mice lacking the c-mer membrane tyrosine kinase, and in C1q complement deficient mice have been shown to lead to lupus like autoimmunity (Cohen *et al.*, 2002 ; Taylor *et al.*, 2000). Recent studies have also shown that impaired uptake of apoptotic cells by macrophages can result in their association with immature dendritic cells (Baumann *et al.*, 2002). The dendritic cells can then phagocytose the apoptotic cells and present apoptotic cell-derived antigens to T cells and B cells (Albert *et al.*, 1998; Inaba *et al.*, 1998; Rovere *et al.*, 1998) .

These results give weight to the notion that apoptotic cells provide an immunogenic stimulus for autoimmunity, and that impaired clearance is an important factor in the pathogenesis of systemic autoimmune disease such as SLE.

1.10 : Double Negative T cells

In a general context, human peripheral blood T cells are classified into T helper (Th) cells which express the surface glycoprotein CD4 and cytotoxic/suppressor cells expressing the surface glycoprotein CD8. Other minor T cell subsets that possess various distinct functional properties and disease associations are –

- "double positive"(DP) T cells expressing both CD4 and CD8 surface glycoproteins and

- "double negative"(DN) cells which express neither the CD4 nor CD8 glycoprotein, but possess a functional T cell receptor (TCR) and the CD3 surface T cell marker.

These double negative T cells can express a T cell antigen receptor consisting of alpha-beta ($\alpha\beta$) chains or gamma-delta ($\gamma\delta$) chains (Bachmaier *et al.*, 1996; Groh *et al.*, 1989). Although the majority of double negative (DN) T cells express the $\gamma\delta$TCR a small proportion of the peripheral $CD4^-CD8^-$ T lymphocytes, express the classical $\alpha\beta$ TCR heterodimer.

1.10.1: αβTCR$^+$ double negative T cells

Double negative, αβ T cell antigen receptor (TCR) expressing cells have been detected in man and mouse and account for a small but variable proportion (1-10 %) of circulating T cells (Kusunoki *et al.*, 1992). The emerging picture of these cells in normal tissue, autoimmune lesions and transgenic mice seems heterogenous, although some common themes related to questions of self-tolerance and self reactivity in the T cell compartment appear frequently. (table 1.7).

αβTCR$^+$ DN T cells can be unambiguously classified as mature T cells by their stable peripheral phenotype (CD2$^+$, CD5$^+$, CD7$^+$) (Londei *et al.*, 1989; Niehues *et al.*, 1994). They lack the expression of CD4 or CD8 glycoproteins: these proteins are neither found on the cell surface nor are their transcripts detectable in these T cells (Rivas *et al.*, 1990; Huang and Crispe, 1992). They are CD3$^+$, CD2$^+$ve and express the IL-2 receptor on their surface, as shown by studies on both stable cell lines and freshly isolated cells from peripheral blood (Brooks *et al.*, 1993; Niehues *et al.*, 1999). They were also found to show high constitutive expression of HLA-DR, a marker for recent T cell activation (Brooks *et al.*, 1993; Niehues *et al.*, 1999; Niehues *et al.*, 1994). A study by Brooks et al, showed the presence of significantly more DN T cells expressing the CD45RO phenotype and low levels of CD45RA and lacking L-selectin, defined as a 'memory' T cell phenotype (Brooks *et al.*, 1993; Niehues *et al.*, 1994). Expression of CD28, a T cell marker up-regulated by activation and survival signals was earlier reported to be absent on αβ DN T cell lines, though a more recent study shows normal expression levels (Niehues *et al.*, 1999; Londei *et al.*, 1989).

	Mouse	Human
A. Normal tissue		
thymus	(Fuss *et al.*, 1997)	
	(Toribio *et al.*, 1988)	
bone marrow	(Dejbakhsh-Jones *et al.*, 1995)	
peripheral blood	(Rivas *et al.*, 1990),	
	(Londei *et al.*, 1989),	
	(Murison *et al.*, 1993),	
	(Niehues *et al.*, 1999)	
spleen	(Reimann, 1991),	
	(Prud'Homme *et al.*, 1991)	(Rivas *et al.*, 1990)
lymph node	(Fowlkes *et al.*, 1987),	
	(Huang and Crispe, 1992)	
skin		(Groh *et al.*, 1989)
B. Autoimmune diseases		
• Human		
Autoimmune lymphoproliferative syndrome (ALPS)		(Fuss *et al.*, 1997),
		(Illum *et al.*, 1991)
Insulin dependent diabetes mellitus (IDDM)		(Wilson *et al.*, 1998)
Systemic sclerosis (SSc)		(Sakamoto *et al.*, 1992)
Autoimmune thyroid disease (AITD)		(Wu *et al.*, 1994)
Systemic lupus erythematosus (SLE)		(Shivakumar *et al.*, 1989),
		(Devi *et al.*, 1998),
		(Liu *et al.*, 1998)

	Mouse	Human
• Murine		
NZB mouse	(Datta *et al.*, 1987),	
	(Adams *et al.*, 1990)	
lpr/lpr mouse	(Benihoud *et al.*, 1997),	
	(Christianson *et al.*, 1996),	
	(Giese and Davidson, 1995),	
	(Ravirajan *et al.*, 1996)	
gld/gld mouse	(Theofilopoulos *et al.*, 1989)	
	(Cohen and Eisenberg, 1992)	
transplanted nude mouse	(Sakaguchi *et al.*, 1985)	

Table 1.7: Double negative ($CD3^+CD4^-CD8^-$) TCR αβ expressing peripheral T cells : distribution in humans and mice. Table adapted from a review by Reimann. (Reimann, 1991).

1.10.2: Functional characteristics of double negative T cells

The nature and function of double negative T cells have not been fully characterised, although they are being extensively studied in view of their associations with disease pathogenesis. αβ TCR^+ DN T cells exhibit a functional T cell receptor (TCR) of limited heterogeneity and are biased towards the preferential usage of certain TCR-Vβ genes both in normal subjects and some disease conditions such as SLE and Systemic Sclerosis (Brooks *et al.*, 1993; Sakamoto *et al.*, 1992; Shivakumar *et al.*, 1989). Various immunological functions are induced in αβ TCR^+ DN T cells by stimulation of CD3-dependent pathway. Unusual patterns of cytokine expression of normal DN T cell subset are not characterised. An unexpected characteristic of these cells was their response to IL-3, a growth factor previously shown to affect haemopoietic cells, chiefly progenitor cells and mast cells (Londei *et al.*, 1989).

Recent reports in literature (reviewed below) have attributed three distinct immunological effector functions to αβTCR$^+$ DN T cells. They are-

- Helper T cell function
- Cytotoxic T cell functions and
- Suppressor/regulatory T cell function.

1.10.3: αβTCR$^+$ DN T cells: Helper T cell function.

αβ TCR$^+$ DN T cell cell clones from healthy individuals have been demonstrated to possess the physiological potential to induce polyclonal immunoglobulin production by autologous B cells (Pelkonen *et al.,* 1990). T helper cell function has also been demonstrated for DN T cells in the context of the autoimmune diseases Autoimmune lymphoproliferative syndrome (ALPS) and SLE (Fuss *et al.*, 1997; Shivakumar *et al.*, 1989). Shivakumar et al., have reported the presence of a αβ$^+$ DN T cell line derived from the peripheral blood of a patient with active SLE that induced the production of pathogenic anti-DNA antibodies by autologous B cells (Shivakumar *et al.*, 1989). Moreover, murine αβ$^+$ DN T cells with helper function for B cells have been identified from the spleens of MRL lpr/lpr and (NZBxNZW)F1 and (NZBxSWR)F1 mice which are known to develop autoimmune manifestations(Datta *et al.*, 1987; Seman *et al.*, 1990; Pelkonen and Palacios, 1990). Various studies have also shown αβ$^+$ DN T cell lines and clones from healthy subjects to have a higher proportion of IL-4 producing clones. These findings have led to the suggestion that these cells may play a role in 'Th2 like' responses, and provide help for antibody production (Katsikis *et al.*, 1995; Niehues *et al.*, 1999). The role of αβ$^+$ DN T cell function as T helper cells is significant in view of their associations with a number of autoimmune diseases.

1.10.4: Cytotoxic activity of αβ TCR$^+$ DN T cells.

Both spontaneous cytotoxicity and cytotoxicity induced by CD3 MoAb (monoclonal antibody) or lectin have been reported for αβ TCR$^+$ DN T cells, although their target antigens and cell surface structures recognising target cells have remained obscure. Spontaneous and MHC unrestricted cytotoxity of αβ$^+$ DN T cells from normal human skin, and the blood of a patient with combined immunodeficiency disease have been demonstrated, probably through specific recognition of the CD1 family of proteins (Groh *et al.*, 1989; Brooks *et al.*, 1990;

Matsumoto *et al.*, 1991; Porcelli *et al.*, 1992). Other contradictory studies report the lack of spontaneous cytotoxic activity, which could be induced in the presence of lectin or anti-CD3 MoAb (Londei *et al.*, 1989). MHC-restricted alloantigen specific αβ TCR^+ DN T cells from peripheral blood of healthy donors have been generated by Rivas et al., suggesting that this population of cells might be heterogeneous with respect to function and MHC restriction (Rivas *et al.*, 1990). The characteristic of any particular clone probably varies with the tissue source and methods of selection/propagation.

1.10.5: Suppressor/ regulatory cell function of αβ TCR^+ DN T cells

Down regulation of immune responses by regulatory T cells is one of the major mechanisms involved in the induction of tolerance to self and alloantigens as demonstrated in a number of models of transplantation and autoimmunity. αβ TCR^+ DN T cells may represent a novel suppressor/regulatory cell population whose function is to regulate immune responses negatively. Reports in the literature have demonstrated a regulatory role for αβ TCR^+ DN T cells in murine models of transplantation tolerance (Young *et al.*, 2002; Young and Zhang, 2002; Carroll, ; Ford *et al.*, 2002). In these studies αβ TCR^+ DN T cells have been shown to specifically eliminate syngeneic $CD4^+$ and $CD8^+$ T cells that share the same TCR specificity as the DN T cells (Zhang *et al.*, 2001).

Mature $CD4^-CD8^-$ $\alpha\beta^+$ T cells (DN T cells) in the periphery of TCR transgenic mice have been found to be resistant to clonal deletion in cognate Ag-expressing (Ag^+) mice (Reimann, 1991). Self-antigens appear to mediate the peripheral expansion and conversion of these cells into potent immunoregulatory cells in the TCR transgenic mice. These self-reactive regulatory cells could be uniquely poised to suppress immune responses and prevent autoimmunity.

In humans, αβ TCR^+ DN T cells bearing the $V\alpha24J\alpha Q^+$ TCR have been found to be in lower frequencies in patients with atopic diseases (Oishi *et al.*, 2000). This sub-population of cells has also been shown by others to be reduced or dysfunctional in patients with several autoimmune disorders such as SLE, systemic sclerosis (Sumida *et al.*, 1995; Oishi *et al.*, 2000; Wilson *et al.*, 1998).

Taken together, these results strongly point to an immunoregulatory role for all or part of the αβ TCR^+ DN T cell population.

1.10.6: Developmental origin of αβ TCR$^+$ DN T cells

αβ TCR$^+$ DN T cells may be the progeny of a unique developmental pathway which either originates from a separate lymphoid stem cell pool, or represents a side-line of one of the two main lineages (TCRαβ$^+$ and TCR γδ$^+$) of T cell differentiation. Thymic as well as an extrathymic developmental origin of αβ TCR$^+$ DN T cells in normal, autoimmune and transgenic mice has been proposed. Different differentiation pathways may be involved, as these cells may represent a heterogeneous cell population.

Thymus dependent development on αβ TCR$^+$ T cells has been suggested by some studies. Thymectomy eliminates the accumulation of αβ TCR$^+$ DN T cells in lpr/lpr and gld/gld mice and DN T cells expressing transgenic TCR in transgenic mice are found to be thymus dependent (Steinberg *et al.*, 1980; von Boehmer *et al.*, 1991). In addition, self reactive αβ TCR$^+$ DN T cells are not found in nu/nu mice (Reimann, 1991).

An extrathymic T cell developmental pathway is suggested by the appearance of TCR expressing CD3$^+$ T cells in athymic nude mice (Reimann, 1991). Extrathymic maturation of αβ TCR$^+$ DN T cells from haemopoietic stem cells has also been demonstrated in thymectomized and irradiated mice, reconstituted with haemopoietic stem cells from the bone marrow of normal mice (Dejbakhsh-Jones *et al.*, 1995). A recent report on a unique population of αβ TCR$^+$ DN T in the mouse female genital tract has indicated these cells are of extrathymic origin as they were found in nude mice as well as mice deficient for MHC class II, β_2 microgobulin and CD1 (Johansson et al., 2003). Studies in mice have also shown that IL-3, a growth factor affecting haemopoietic cells could induce differentiation and/or proliferation of αβ TCR$^+$ DN T cells in the bone marrow (Kubota *et al.*, 1992). The appearance of DN T cells bearing self-reactive T cell receptors in the livers of mice injected with bacteria might indicate the liver as a potential site for a major extrathymic pathway for T cell differentiation (Abo *et al.*, 1991).

1.11: αβ TCR+ve DN T cells: do they have a role in SLE?

DN T cells are notorious for their involvement in murine lupus of MRL/lpr and gld/gld mice, which have been used as a model of human systemic lupus erythematosus (SLE). Massive numbers of DN T cells accumulate in the secondary lymphoid organs including lymph nodes and spleen in lupus prone MRL/lpr and gld mice (Cohen and Eisenberg, 1992). Many of the autoreactive T cell clones isolated from the MRL/lpr mice were found to be lacking expression of CD4 and CD8 co-receptors. All these autoreactive T cells responded to syngeneic splenic cells and helped syngeneic B cells to produce anti-DNA antibodies, especially of the IgG class (Chen *et al.*, 1997). Similar autoreactive helper DN T cells have also been isolated from the (SWRxNZB) F1 (SNF1) murine model of lupus (Adams *et al.*, 1990; Datta *et al.*, 1987). These observations have highlighted a possible role for DN T cells in SLE.

In human SLE, Shivakumar et al (1989) first reported that the markedly expanded αβ TCR^+, $CD3^+$ T cells, among the $CD^-/CD8^-$ population from active lupus contained the double-negative subset of T helper cells (Th) responsible for augmenting the production of pathogenic autoantibodies. Two later studies reported an expanded population of αβ TCR^+ DN T cells in patients with active SLE, but the increased numbers were not always statistically significant (Devi *et al.*, 1998; Liu *et al.*, 1998). Increased levels of total DN T cells (expressing αβ or γδ TCR) have also been reported in patients with SLE, with numbers decreasing after six months of cyclophosphamide treatment and better clinical prognosis (Lacki *et al.*, 1997). A recent study by Sieling et al., suggested that CD1 restricted DN T cells from patients with SLE can provide help to $CD1^+$ B cells for IgG antibody production and could therefore promote pathogenic autoantibody responses in SLE (Sieling *et al.*, 2000). Thus, although there is evidence of DN T cells playing a role in the pathogenesis of SLE, the mechanisms of their contribution remain unclear and merit detailed study.

Preliminary work on DN T cells using two-colour immunofluorescence staining in our laboratory indicated an increased expression of the Bcl-2 anti-apoptotic protein in the DN T cell population of patients with SLE compared to the same population in healthy subjects. In addition, other investigators have shown that DN T cells from patients with SLE provide T cell help to autologous B cells augmenting the production of pathogenic autoantibodies. Based on these observations, we hypothesised that DN T cells in patients with SLE could, perhaps survive for unusually long periods of time (resisting activation induced cell death) due

to an overexpression of Bcl-2 protein, resulting in expanded populations that contributed to the disease pathogenesis by providing prolonged T cell help to autoreactive B cells. Therefore, we undertook to study activation status, Th2 associated cytokine (IL-4) levels and apoptosis susceptibility in DN T cells from patients with SLE and healthy individuals to establish their role in the disease pathogenesis of SLE.

1.12: Major Aims of this Thesis:

1. To determine the levels of DN T cells in patients with SLE.
2. To determine the activation status of DN T cells in patients with SLE.
3. To determine the production of the Th2 associated cytokine IL-4 by DN T cells in patients with SLE.
4. To investigate the susceptibility of SLE DN T cells to apoptosis.

Chapter 2.

Materials and Methods.

2.1:Materials.

2.1.1:Reagents.

Heparin BP	Monoparin: CP Pharmaceutical, Clwyd, UK.
RPMI 1640	Life Technologies, Renfrewshire, UK.
Ficoll hypaque	Nycomed, Birmingham, UK.
Hank's Balanced salt solution (HBSS)	Life Technologies,Renfrewshire, UK.
Foetal calf serum	Life Technologies, Renfrewshire, UK.
Penicillin and Streptomycin (Pen/Strep)	Life Technologies, Renfrewshire, UK.
Glutamine	Life Technologies, Renfrewshire, UK.
Acridine Orange	Raymond A. Lamb. Wembley, London, UK.
Ethydium bromide	Sigma chemical Co. Dorset, UK.
Dynabeads M-450 (sheep anti-mouse IgG	Dynal, Norway.
Bovine serum albumin	Sigma chemical Co. Dorset, UK.
Sodium azide	Sigma chemical Co. Dorset, UK.
Rabbit anti-mouse immunoglobulin (fluorescein isothiocyanate comjugate)	DAKO Ltd. Buckinghamshire, UK.
Rabbit anti-mouse immunoglobulin (phycoerythrin conjugate)	DAKO Ltd. Buckinghamshire, UK.
Paraformaldehyde	Merck Ltd, Leicestershire, UK.
Normal mouse serum	DAKO Ltd. Buckinghamshire, UK.
Human Immunoglobulin G (Cohn fraction II)	Sigma chemical Co. Dorset, UK.
Permeafix™	Ortho, New Jersey, USA.
Apoptosis Detection Kit	R&D systems, Minneapolis, USA.
Lysine coated slides	Sigma chemical Co. Dorset, UK.
Mounting fluid	Vecta laboratories, Burlingame, USA.
Caltag Fix and Perm	Caltag laboratories, Burlingame, USA
Phytohaemagglutinin (PHA)	Sigma chemical Co. Dorset, UK.
Monensin	Sigma chemical Co. Dorset, UK.

Phosphate buffered saline (PBS) tablets	Sigma chemical Co. Dorset, UK.

2.1.2: Monoclonal Antibodies.

2.1.2.1: Hybridomas.

UCHT-8	anti-human CD4	a kind gift from Prof. P. Beverly.
UCHT-4	anti-human CD8	a kind gift from Prof. P. Beverly.
BU-12	anti-human CD19	a kind gift from Prof. J. Gordon.
MY-23	anti-human CD14	a kind gift from Prof. M. Fanger.
UCLH-1	anti-human CD45R0	a kind gift from Prof. B.M. Chain.

2.1.2.2: Directly conjugated antibodies.

Anti-CD3 FITC	Pharmingen, SD, U.S.A.
Anti-CD4 CyChrome	Pharmingen, SD, U.S.A.
Anti-CD8 CyChrome	Pharmingen, SD, U.S.A.
Anti-αβTCR FITC	Pharmingen, SD, U.S.A.
Anti-γδ TCR FITC	T cell Diagnostics, Cambridge.
Anti-CD69 FITC	Pharmingen, SD, U.S.A.
Anti-HLA-DR FITC	DAKO Ltd. Buckinghamshire, UK.
Anti-CD28 FITC	a kind gift from Dr. Y. Latchmann.
Anti-Fas FITC	DAKO Ltd. Buckinghamshire, UK.
Anti-IL-4 PE	R&D systems, Minneapolis, USA.
Anti-Bcl-2 FITC	DAKO Ltd. Buckinghamshire, UK.

2.1.2.3: Un-conjugated antibodies.

Anti-CTLA-4	a kind gift from Dr. B. Bröker.
Anti-Bcl-x	Santa Cruz biotechnology, U.S.A.
Anti-bax	Santa Cruz biotechnology, U.S.A.
CD45 RA	a kind gift from Prof. B.M. Chain.+

2.1.2.4: Isotype controls.

IgG1 FITC	DAKO Ltd. Buckinghamshire, UK.
IgG1 PE	Pharmingen, SD, U.S.A.
IgG1 CyChrome	Pharmingen, SD, U.S.A.
$IgG2_b$ FITC	Pharmingen, SD, U.S.A.

2.1.3: Plastics.

All plastics were supplied by Life Technologies, Renfrewshire, UK.

2.2: Methods.

2.2.1: Cell source.

2.2.1.1: Patients and Controls.

Peripheral blood was obtained from patients with Systemic Lupus Erythematosus attending the Middlesex Hospital Rheumatology clinic with informed consent. Each met four or more of the revised criteria of the American college of Rheumatology for the classification of rheumatic disease (Tan *et al.*, 1982). Disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) computerised index (Hay *et al.*, 1993). The index is based on the 'physician's intention to treat' principle and divides lupus activity into eight organs or systems which are scored from A (most active) to E (never previously active). A global score determined using A=9, B=3, C=1, D=0, E=0 has been successfully compared with other methods of calculating global scores (Gladman *et al.*, 1994), and in this study patients scoring >6 were deemed active and <6 inactive. Patients with SLE were predominantly female (1male patient studied), with an average age of 35 and ranging from 17 to 50 years of age. Levels of circulating anti-dsDNA antibodies and C3 levels were measured during routine patient assessment. Serum levels of anti-dsDNA antibodies in excess of 50 IU/ml(ELISA test-Shield diagnostics, Dundee) and levels of C3 less than 0.9 IU/ml(by laser nephelometry) are regarded as abnormal. For statistical analysis in this study, anti-dsDNA antibody levels of ≥100 IU/ml were considered to be high. Patients with Rheumatoid Arthritis (RA) who fulfilled four or more of the ARA criteria for Rheumatoid Arthritis and age and sex matched healthy controls were also studied (Arnett *et al.*, 1988). Details of relevant patient groups are given in the appropriate results chapters. Control blood was taken from healthy laboratory staff and medical students (with no history of autoimmune disease).

2.2.1.2: Preparation of human peripheral blood mononuclear cells using density gradient separation.

Peripheral blood samples (50mls) were collected in heparinised (100 Units) Universals by venesection. Blood samples from patients and age and sex matched healthy control volunteers were collected with informed consent. Peripheral blood was diluted 1:1 in RPMI 1640 and 25ml carefully layered over 25ml of Ficoll-Hypaque Lymphopaque 1077. The density gradient and diluted blood were spun at 2000rpm(750g) for 30 minutes at room temperature. After spinning, the interface of cells between the diluted blood and Ficoll-Hypaque was aspirated using a sterile Pasteur pipette and washed twice in Hank's Balanced Salt Solution (HBSS) at 1500rpm, 4°C for 10'. The resulting cell pellet was suspended in a known volume of medium and 20µl stained with an equal volume of Ethidium Bromide Acridine Orange solution . Twenty microlitres (µl) of this mixture was introduced under the coverslip of an Neubauer haemocytometer and the number of cells and viability assessed using a fluorescence microscope. The viable cells stained green and the non-viable cells orange. Cell viability was calculated by dividing the number of viable cells (green) by the total number cells in the field (green +orange cells). A greater than 95% viability was obtained in most of the samples after density gradient separation.

2.2.2: Enrichment of Double Negative T cells.

The double negative T cells in the PBMC's were enriched by negative depletion using Dynabeads. Separated Mononuclear cells were incubated with monoclonal antibodies raised against CD4, CD8, CD19, and CD14. The monoclonal antibodies were in the form of supernatants from Hybridoma cultures, anti-CD4 from the hybridoma UCHT8, anti-CD8 from UCHT4, anti-CD19 from BU12 and anti-CD14 from MY23. Each of the four supernatants from the hybridomas were added to the cells at super-saturation levels (1.5 ml each) and incubated on ice for 30' with gentle mixing. The unbound antibodies were then washed off with an excess of RPMI 1640 at 1500 rpm, 4°C for 10'. Dynabeads (sheep anti-mouse IgG) were washed twice, by placing the required amount in a polypropylene tube (15ml) on the magnet and removing the unattached liquid and reconstituting the Dynabeads bound to the magnet with an equal volume of RPMI1640 as was removed. The washed Dynabeads were added to the cells with previously bound surface antibody to a final concentration of 1-2 x

10^7/ml and with at least 4 Dynabeads for each cell. The mixture was incubated for 30' on a roller at 4°C. The rosetted cells (bound to beads) were removed by placing the tube on the magnet and aspirating the supernatant containing unbound cells. The aspirated cells were washed in excess volumes of complete medium (RPMI 1640+10% Fetal calf serum) at 1500rpm and 4°C for 10'. The cell pellet was suspended in an appropriate volume of complete medium for cell culture or staining. The enrichment was checked by immunofluoresence staining and flow cytometric analysis (described in detail in chapter 3) and was always greater than 95% pure.

2.2.3: Immunofluorescence staining and flow cytometry.

Separated mononuclear cells or enriched double negative cells were re-suspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.05% sodium azide (NaN_3), at $1x10^6$ cells/ml. Two hundred µl of this cell suspension was added to each well of a 96 well U bottomed tissue culture plate and spun at 1500 rpm for 5'. The supernatant was aspirated, the cell pellet re-suspended by agitating the plate and 20µl of appropriate monoclonal antibody added to each well at the required dilution. The cells were incubated with the antibody for 40' on ice. The cells were washed 3 times with PBS/BSA, by adding 200µl to each well and centrifuging the plate as before and aspirating the supernatant. A 1:20 dilution of the second antibody, a rabbit-anti mouse immunoglobulin conjugated to a relevant fluorochrome, was made up in PBS/BSA and 20µl added to the resuspended cell pellet. The cells were incubated for 40' on ice and again washed 3 times with PBS/BSA. After the final wash, the cells were re-suspended in 200µl of 1% paraformaldehyde in PBS. The cells were left for at least one hour before analysis on a FACScan flow cytometer (Becton Dickinson). If the cells were to be left overnight the plate was wrapped in aluminium foil to exclude light and kept at 4°C until analysed.

2.2.3.1: Double Immunofluorescence staining of cells for flow cytometric analysis.

The mononuclear cells were treated for the first part as above and stained with a first, and fluorochrome conjugated second antibody. After washing 3 times to remove any unbound second antibody, the cell pellet was re-suspended and 20µl of a 2% normal mouse serum (NMS) was added and the cells incubated on ice for 40'. The mouse serum was added to

prevent any free binding sites in the secondary antibody to bind non-specifically to final antibody. The cells were again washed 3 times in PBS/BSA and 20µl of final antibody directly conjugated to a complementary fluorochrome, was added to the re-suspended cells and incubated on ice for 40'. After a final 3 washes the cells were fixed with 2% paraformaldehyde and analysed on a flow cytometer as described above.

2.2.3.2: Three colour immunofluorescense staining.

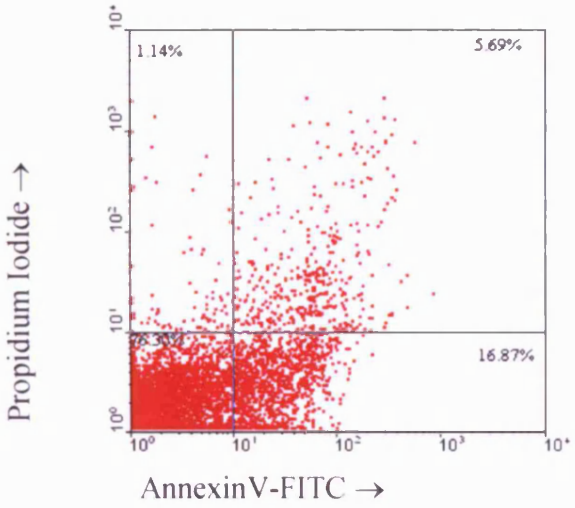
Cells were stained with CyChrome™ (Pharmingen) and PE conjugated monoclonal antibodies with the third colour being FITC. Cells were incubated with 20µl of a cocktail of antibodies containing CD3-PE (FL2 channel), CD4-CyChrome, and CD8-CyChrome (FL3 channel) in the ratio 1:2:2, as previously titrated for optimum staining. A third antibody conjugated to FITC and fluorescing in the FL1 channel was also added to the cells at concentrations for optimum staining.

2.2.3.3: Intracellular staining for Bcl-2 family of proteins.

The Bcl-2 (Bcl-2, Bax, Bcl-x) family of proteins are localised in the cytoplasm of the cells. Hence it is necessary to permeabilise the cells to enable the antibodies to enter the cells and bind to the antigen. To enable this, cells were incubated with 50µl of 1:1 dilution in PBS of ORTHO Permeafix™, a one step permeabilising and fixative reagent and incubated for 40' on ice. The cells were then washed 3 times in PBS/BSA and stained with anti-Bcl-2, anti-Bax or anti- Bcl-x antibodies as described above.

2.2.4: Measurement of apoptosis.

An AnnexinV-FITC/Propidium Iodide kit was used to quantitate levels of apoptosis in cell samples. The cells were washed in 100µl annexin binding buffer (supplied with the kit) at 1500 rpm for 5'. Twenty µl of annexin binding buffer was added to the cells in each well along with 5µl of AnnexinV-FITC and 5µl of Propidium iodide . The cells were then incubated in the dark at room temperature for 15 minutes before being analysed on the FACScan (Becton Dickinson). Representative dotplot of AnnexinV-FITC/Propidium Iodide staining are shown below.



2.2.5: Flow cytometric analysis of mononuclear cells.

The stained cell preparations were analysed on a Becton Dickinson FACScan flow cytometer using Win FCM research software. Essentially, the mononuclear cells were gated on forward light scatter (FSC) and right angle light scatter (SSC) to discount any debris or polynuclear cells. The cells were analysed for fluorescence staining on channels FL1 (FITC), FL2 (PE) and FL3 (Cy chrome). Whenever possible, a minimum of 10,000 events was analysed. Data analysis was performed using WinMDI version 2.8 software.

2.2.6: Confocal Microscopy of stained cells.

The cells were stained as described above and fixed in 1 % paraformaldehyde. The cells were then placed on a Lysine coated slide and incubated for 10 minutes. The non-adherent cells were removed and the slide coated with mounting fluid and covered with a cover slip. The slides were then analysed under a confocal microscope (MRC 1024 [Bio-Rad] equipped with a krypton-argon laser). The confocal-assistant software was used to analyse data and generate images.

2.2.7: T cell activation assays.

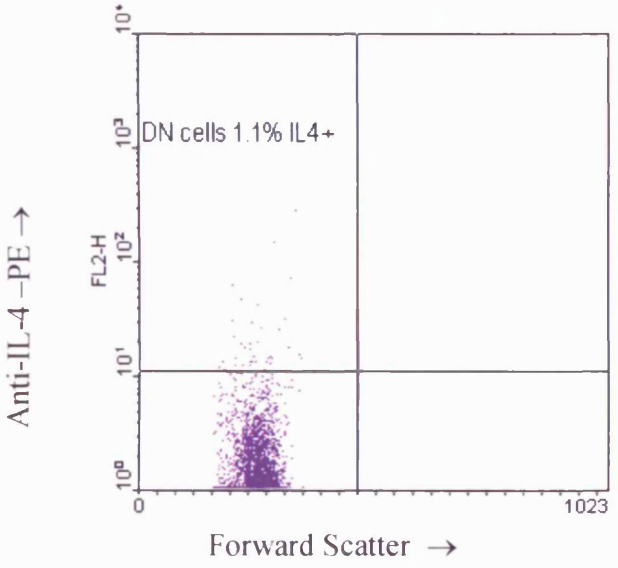
Enriched double negative T cells or $CD4^+/CD8^+$ T cells were cultured in sterile NUNC 24 well plates in complete medium (RPMI 1640 + 10% fetal calf serum) supplemented with

L-glutamine (100U/ml), penicillin (100U/ml) and streptomycin (100μg/ml) at 37°C, 5% CO_2. Cells were stimulated by adding a 20% stock solution of PHA in complete medium to each well, such that the final concentration was 1%. Cells were harvested at appropriate time points, washed and immunofluoresence staining for activation markers was carried out as described above.

2.2.8: Intracellular staining for IL-4.

2.2.8.1: Constitutive levels of IL-4.

PBMCs from patients with SLE and healthy controls were fixed and permeabilized using Caltag Fix and Perm in accordance with the manufacturer's instructions prior to intracellular staining for IL-4. Background staining was determined using mouse isotype and conjugate-matched irrelevant antibodies. Representative dotplot of intracellular IL-4 staining of enriched DN T cells is shown below.



2.2.8.2: IL-4 levels after PHA stimulation.

PBMCs from patients and controls were incubated overnight at 37° C, 4.5% CO_2 in the presence of 10 μg/ml PHA. Monensin (0.05M) was added for the final 6 hours to facilitate accumulation of cytokine in the Golgi and the cells were stained for surface markers and intracellular IL-4 as described above.

2.2.9: Statistical analysis.

Data were analysed using the non-parametric Mann-Whitney test and the Wilcoxon's test for paired samples. The two-tailed non-parametric (Spearman) test with 95° confidence limits was used to determine correlations.

Chapter 3.

DN T cells in patients with SLE.

3.1: Introduction and aims.

αβ TCR+ve Double negative T cells have been shown to be associated with the pathogenesis of systemic lupus erythematosus in previous studies. This chapter aims to study the presence and frequency of double negative T cells in the peripheral blood of patients with SLE compared to patients with RA and healthy individuals.

3.1.1: Introduction.

The exact role of double negative (DN) T cells in the pathogenesis of systemic autoimmune diseases such as SLE has not been established. However, studies in recent years have indicated a firm link between the dysregulation of the frequency and function of this T cell population with a number of autoimmune diseases including SLE.

The DN T cell subset in man was identified in the late 1980's, although the exact nature and role of this enigmatic T cell subset is not yet fully understood (de la Hera *et al.*, 1985). Double negative T cells have been shown to express either the αβ or γδ TCR (Shivakumar *et al.*, 1989; Rajagopalan *et al.*, 1990). Double negative T cells by definition, lack the CD4 and CD8 adhesion receptors of the immunoglobulin superfamily which participate in specific antigen recognition of T lymphocytes by acting as co-receptors for MHC class I and II molecules (Parnes, 1989; Springer, 1990). CD4 and CD8 molecules also mediate signal transduction in the course of T cell activation (Barber *et al.*, 1989). The prominent role of the CD4 and CD8 co-receptors in T cell function makes it surprising to find DN T cells in peripheral tissues of mouse and man. Hence, it has been suggested that these cells may not be restricted by the major histocompatability complex molecules (MHC) (Sieling, 2000). Investigators have also suggested a role for non-classical MHC –like molecules, the CD1 family of antigen presenting proteins.

Association of DN T cells with various autoimmune diseases such as systemic sclerosis, autoimmune lymphoproliferative disease (ALPS) and SLE argues for their role in the pathogenic process of autoimmune diseases (Sakamoto *et al.*, 1992; Furukawa, 1997; Illum *et al.*, 1991; Rajagopalan *et al.*, 1990; Shivakumar *et al.*, 1989). Double negative T cells (expressing αβ or γδ TCR) are thought to be a separate lineage of T cells and their apparent autoreactive nature may be as a result of avoidance of the process of thymic selection that

would delete the autoreactive T cells. Their lack of CD4 or CD8 co-receptors might aid escape from negative selection in the thymus leading to potentially autoreactive cells in the periphery. In addition, their proposed use of the CD1 antigen presenting molecules to recognize unusual antigens such as hydrophobic lipids and peptides furthers the argument for autoreactivity due to 'antigenic mimicry'. This theory for the activation of self-reactive T cells is that the immune response spreads from first targeting exogenous antigens to targeting highly related self-antigens secondarily (Hayday and Geng, 1997; Barnaba and Sinigaglia, 1997; Schaible and Kaufmann, 2000). DN T cells have been found to be present in all healthy individuals and account for a small but variable proportion (1-10 %) of circulating T cells.

In contrast to human DN T cells, murine DN T cells have been the subject of a number of investigations, most of which have been performed in the lpr/gld mice which develop an SLE/arthritis like autoimmune condition. These studies have revealed a number of unique characteristics of these cells. They are oligoclonal and express normally deleted (forbidden) Vβ regions implying that they have bypassed negative selection in the thymus(Kubota *et al.*, 1992). Furthermore, they bear reduced levels of the CD3: TCR complex and respond to haematopoietic stem cell growth factor IL-3 (Okuyama *et al.*, 1992; Abo *et al.*, 1991; Kubota *et al.*, 1992).

Similarly, one of the characteristic features of human αβ TCR+ve DN T cells from healthy individuals and disease conditions (SLE and adult T cell leukaemia) that has been reported previously, is the lower levels of CD3:TCR complex expressed by these cells *in-vivo* (Shivakumar *et al.*, 1989) (Suzushima *et al.*, 1993; Londei *et al.*, 1989). In contrast, γδ TCR^+ DN T cells from the same individuals expressed CD3: TCR levels similar to those of $CD4^+$or $CD8^+$, indicating that the reduced levels observed in the αβ TCR^+ DN T cell population are not related to the absence of the CD4 or CD8 markers (Murison *et al.*, 1993). Studies on αβ TCR^+ DN T cells in lymph nodes of normal mice have also revealed that the TCR density on the surface of these cells was lower than that of $CD4^+$ and $CD8^+$ T cells (Huang and Crispe, 1992). Although the CD3: TCR complex on αβ TCR^+ DN T cells has been shown to be functionally competent, the significance of lower cell surface densities of the CD3:TCR complex is not known (Groh *et al.*, 1989; Rivas *et al.*, 1990; Matsumoto *et al.*, 1991).

Systemic lupus erythematosus (SLE) is characterised by antibody production against non-protein self-antigens, including nucleic acids and phospholipids. Autoantibody responses

in SLE are T cell dependent, as shown by studies demonstrating a decrease in the incidence of SLE in T cell depleted lupus prone mice and HIV infected individuals (Wofsy and Seaman, 1987; Molina *et al.*, 1995). Insight into the nature of T and B cell interactions in SLE has come in part, from the study of $CD4^-CD8^-$ (double-negative (DN)) T cells. The involvement of expanded populations of DN T cells in murine lupus of MRL/lpr mice, which have been used as a model of human SLE has led to an interest in their role in this autoimmune disease. Massive numbers of DN T cells accumulate in the secondary lymphoid organs including lymph nodes and spleen in lupus prone MRL/lpr mice when proteinuria due to nephritis ensues (Sobel *et al.*, 1993) The frequency of both total DN ($\alpha\beta$ and $\gamma\delta$ TCR^+) T cells and the $\alpha\beta$ TCR expressing sub-population of DN T cells have been shown to be increased in patients with SLE (Shivakumar *et al.*, 1989; Devi *et al.*, 1998; Lacki *et al.*, 1997). The increased numbers have been observed particularly in patients with active disease, suggesting that these T cells are involved in the pathogenesis of the disease. A study of Chinese patients with SLE showed increased levels of $\alpha\beta$ TCR^+ DN T cells within the total lymphocyte population, although the differences did not reach statistically significant levels when compared to healthy individuals (Liu *et al.*, 1998).

T cells expressing the $\gamma\delta$ TCR are attractive candidates for mediators of autoimmunity and have been linked to a number of autoimmune conditions including SLE, Sjögren's syndrome and RA (Brennan *et al.*, 1989; Gerli *et al.*, 1991). Studies on $\gamma\delta$ TCR^+T cells (including both $CD4^+$or $CD8^+$ T cells and DN T cells) have reported both decreased and increased numbers of these cells in the peripheral blood of patients with SLE (Gerli *et al.*, 1991; Riccieri *et al.*, 2000 ; Robak *et al.*, 2001 ; Robak *et al.*, 1999).

Interestingly, in one study reporting decreased numbers of $\gamma\delta^+$ T cells in the peripheral blood, increased numbers of $\gamma\delta$ TCR^+ T cells were detected in clinically normal skin from patients, compared to healthy controls. The accumulation of $\gamma\delta$ TCR^+ T cells in the skin of patients with SLE was also found to have a positive correlation with their disease activity (Robak *et al.*, 2001). The role of the expanded $\gamma\delta$ TCR^+ T cell population in the skin of SLE patients and their relation to disease pathogenesis remains unclear. Pathogenic anti-DNA autoantibody- inducing $\gamma\delta$ TCR^+DN T cells have also been isolated from patients with SLE although expansion in their numbers in the peripheral blood has not been reported consistently (Rajagopalan *et al.*, 1990).

3.1.2: Aims.

In order to understand the role of DN T cells in the pathogenesis of SLE, the frequency of double negative (DN) T cells ($CD3^+$ $CD4^-$ $CD8^-$) expressing αβ TCR and γδ TCR, have been examined in patients with SLE. Patients with rheumatoid arthritis (RA) as autoimmune disease controls, and healthy individuals were also included in the study. Since αβ TCR+ve DN T cells have been previously reported to express significantly lower levels of CD3: TCR complex, the density of CD3 molecules on the surface of DN T cells has also been studied in patients and control subjects.

3.2: Results.

3.2.1: Patients used in this study.

The data in this chapter is based on peripheral blood samples taken from 18 patients diagnosed with SLE and 15 patients diagnosed with rheumatoid arthritis at the Bloomsbury Rheumatology Clinic, UCL. Control blood was taken from 15 healthy laboratory staff and medical students (with no history of autoimmune disease) in the department of Immunology UCL.

The SLE patients all met four or more criteria of the American College of Rheumatology for the classification of the disease (Tan *et al.*, 1982). The mean age of SLE patients was 42 years and the range 17-67 years. Fourteen of the patients were female and one male. Table 3.1 below lists the disease activities of the patients included in this study.

Fifteen patients with rheumatoid arthritis, all of whom met the criteria for the disease as determined by Arnett et al, were studied as autoimmune disease controls (Arnett *et al.*, 1988). The mean age of the cohort of RA patients studied was 59 years, the range 35-82 years and included 11 females and 3 males.

The normal healthy controls (HC) consisted of laboratory staff and students. The mean age was 31 years and the range 18-45 years. Fourteen females and one male were studied.

Patient	Global Score	Drug treatment
1	7	No major drugs
2	4	No major drugs
3	10	Anti- hypertensive only
4	13	Anti- hypertensive only
5	3	200 mg Plaquenil
6	9	400 mg Plaquenil
7	4	400 mg Plaquenil
8	6	15 mg Prednisolone
9	6	15 mg Predisolone
10	5	100 mg Azathioprine
11	4	5 mg Prednisolone 15 mg weekly Methotrexate
12	6	3 mg Predisolone 100 mg Azathioprine
13	3	20 mg Prednisolone 150 mg Azathioprine
14	3	3 mg Prednisolone 100 mg Azathioprine
15	4	5 mg Prednisolone 15 mg weekly Methotrexate
16	6	15 mg Prednisolone 125 mgAzathioprine
17	8	400 mg Plaquenil 8 mg Prednisolone
18	5	400 mg Plaquenil 5 mg Prednisolone 150 mg Azathioprine

Table 3.1: Disease activity (global score) and treatments of patients with SLE studied in this chapter.

3.2.2: Enrichment of Double negative T cells from PBMC.

Mononuclear cells were isolated by density gradient separation from peripheral blood and an enriched population of DN T cells obtained by depletion of $CD4^+$, $CD8^+$ T cells, B cells and monocytes as described in chapter 2. Three-colour

immununofluoresence staining with CD3-PE, CD4-Cy Chrome and CD8-Cy Chrome identified the DN ($CD3^+$ $CD4^-$ $CD8^-$) T cell compartment. A representative FACScan profile of the cell populations studied is shown in Figure 3.1. The enriched DN T cell population was tested for purity and was consistently more than 95% pure.

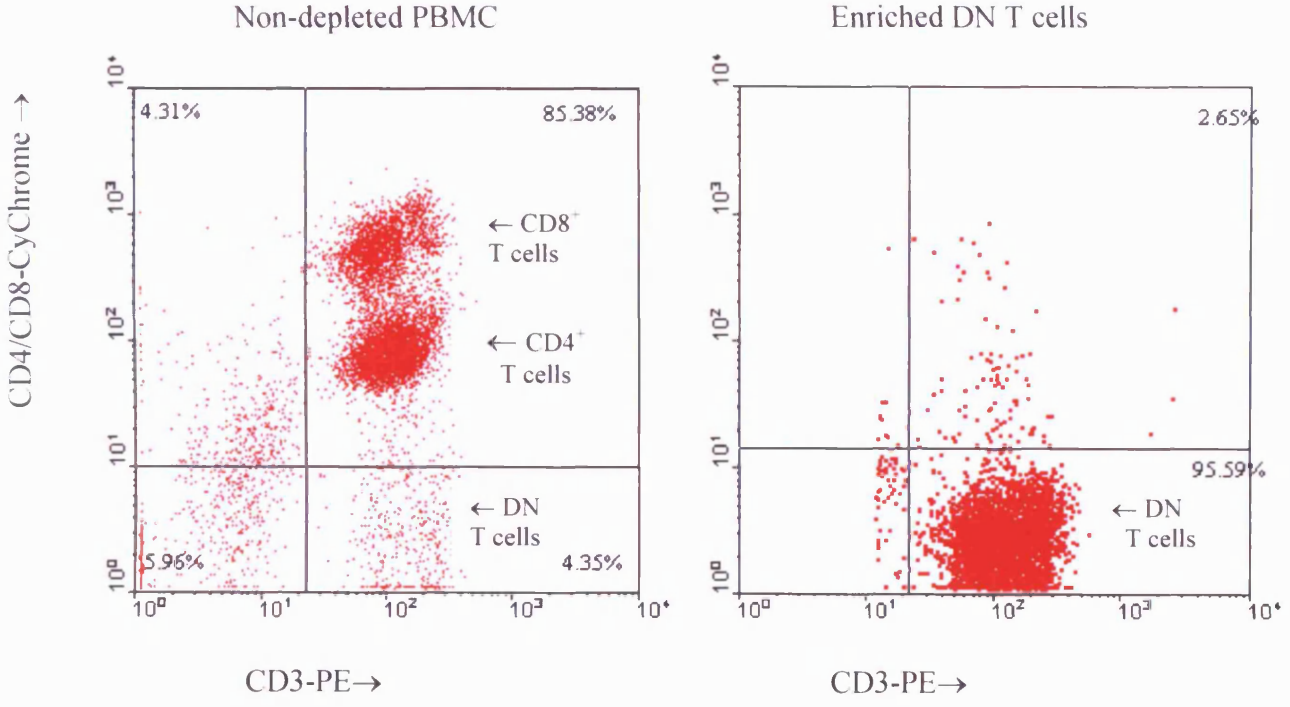


Figure 3.1: Representative fluorescence profiles of lymphoid cells stained for CD3 (horizontal axis), CD4 and CD8 markers (vertical axis), before and after enrichment of double negative (DN) T cells.

3.2.3: Confocal microscopy images of DN T cells.

Enriched populations of DN T cells were stained with antibodies to CD3 (conjugated to the fluorochrome phycoerythrin (PE)) marker and the CD4 and CD8 co-receptors (conjugated to the fluorochrome CyChrome (Cy)) to visualize DN T cells using two colour confocal microscopy as described in chapter 2. This method was used to confirm the correct identification of single positive (SP) and DN T cells using the combination of CD3 and CD4/CD8 set of markers.

Enriched DN T cells stained with anti-CD3 PE antibody (red)

Enriched DN T cells stained with anti-CD4 and anti-CD8 CyChrome antibodies (blue)

Confocal image with DN T cells (red-CD3+CD4/8-) and single positive(SP) T cells (purple-CD3+ CD4 or CD8 +).

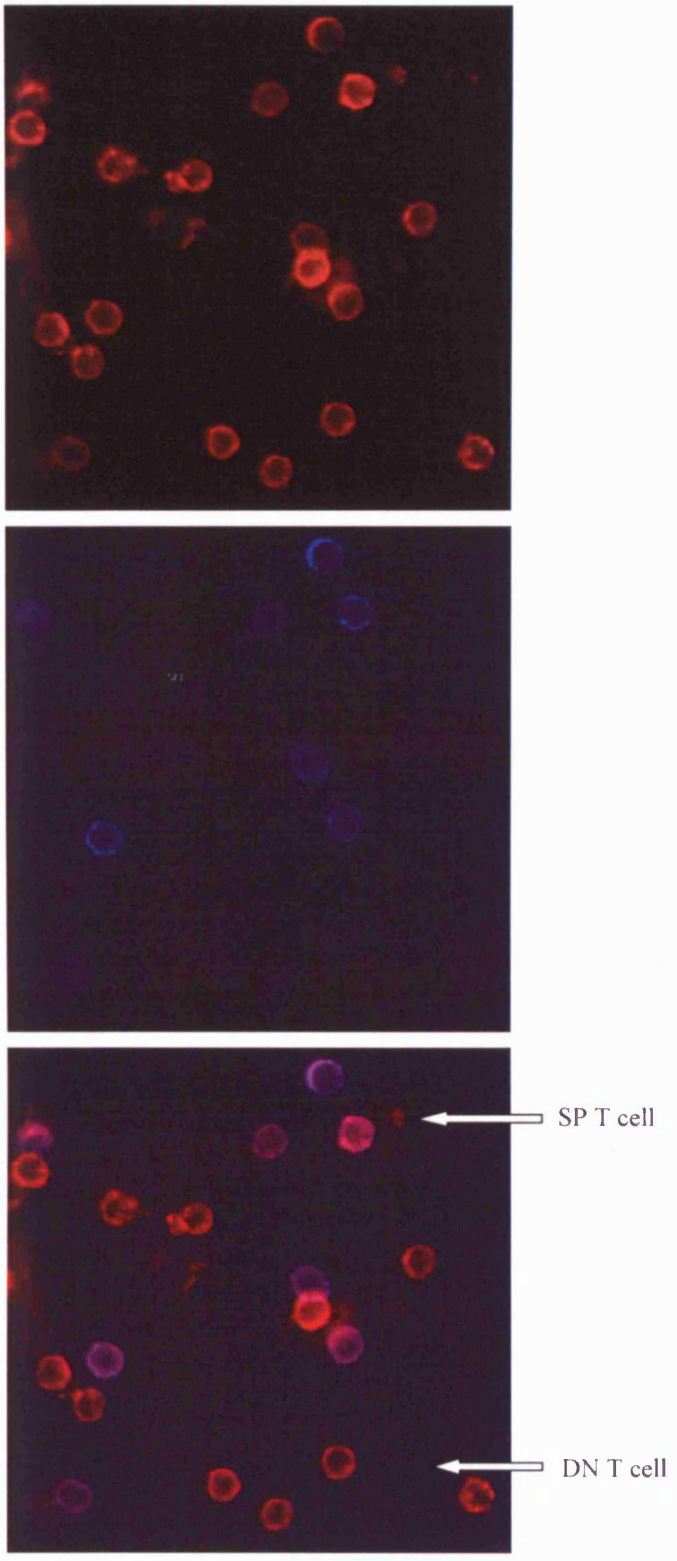


Figure 3.2: Confocal Images of enriched DN T cell populations stained with anti-CD4 and anti-CD8-CyChrome (blue), and anti CD3-PE (red) antibodies. Stained cells were observed by confocal microscopy and images generated using confocal assistant software.

3.2.4: The total T cell population is not expanded in patients with SLE when compared to patients with RA or healthy controls.

The percentage of total T ($CD3^+$) lymphocytes was determined in patients with SLE using flow cytometric staining with an anti-CD3-PE antibody. Using Win MDI software an electronic gate (R1) was placed around the lymphocyte population in a forward scatter (FSC) vs side scatter (SSC) dotplot. Percentage of cells gated on R1 and positive for PE staining (FL2 channel) were quantitated. The results of the investigation are shown in Figure 3.3. As shown in the figure the percentage of total T cells showed no differences between patients with SLE, RA and healthy controls (HC: p=0.4). Overall, SLE patients had lower numbers of lymphocytes compared with healthy controls (SLE: 1.05±0.36x 10^6cells/ ml, HC: 6.45±2.45x10^6cells/ml).

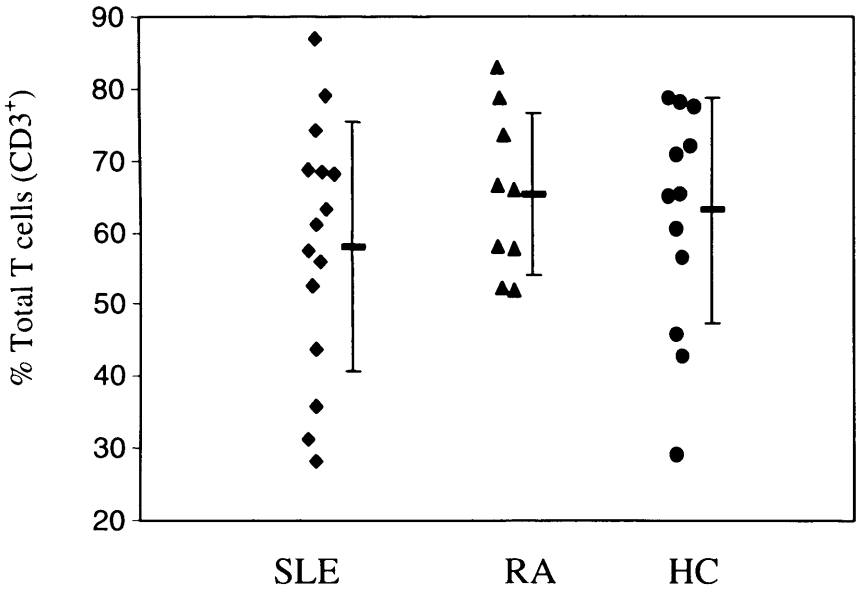


Figure 3.3: Percentage total T cells in SLE (n=15), RA (n= 9) and healthy controls (HC, n=12) showed no significant difference (p= 0.4). Total blood PBMCs were stained with an anti-CD3-PE antibody and analyzed by flow cytometry.

3.2.5: Quantitation of double negative T cells in patients with SLE, RA and healthy controls.

As a start to understanding the contribution of DN T cells to SLE disease pathogenesis, the presence and frequency of DN T cells in the blood of patients with SLE was determined and compared to levels in patients with RA (autoimmune disease controls) and healthy individuals. The proportion of DN T cells in the PBMC's of patients with SLE, RA and HC was measured by two-colour flow cytometry using antibodies to CD3 and CD4 and CD8 as described in section 3.2.2. The percentage of DN T cells in the PBMCs of SLE patients did not differ from autoimmune control patients with RA or healthy controls as shown in Figure 3.4 (SLE:7.7±10.4, RA:4.87±7.12, HC:7.12±4.05) (p=0.3). There was also no correlation between the percentage of DN T cells and the disease activity (as defined by their global score) in patients with SLE (r= - 0.404 and p= 0.89).

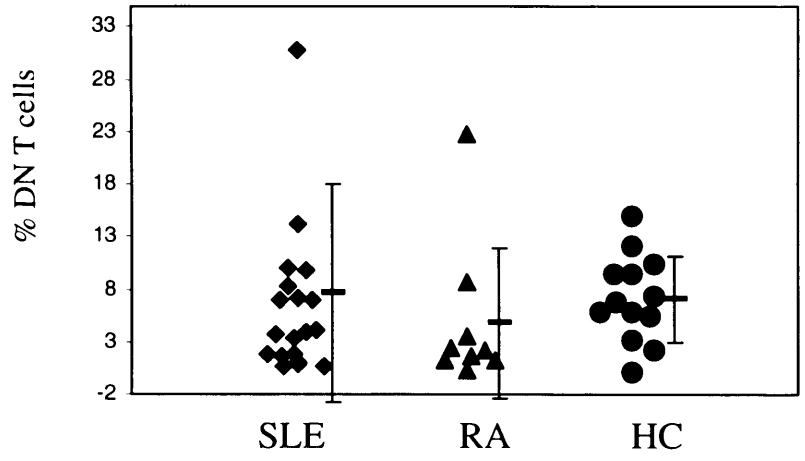


Figure 3.4: The percentages of blood DN T cells in patients with SLE (n=18), RA (n=9) and HC (n=13) did not show statistically significant differences (p=0.3). Total blood PBMCs were stained with anti-CD3-PE (FL2), anti-CD4 and anti-CD8 CyChrome(FL3) conjugated antibodies and analyzed by flow cytometry. DN T cells were defined as $CD3^+$ $CD4^-$ $CD8^-$.

3.2.6: Expanded population of circulating αβ TCR expressing DN T cells in patients with SLE.

The proportions of αβ and γδ T cell receptor-expressing cells in the DN T cell compartment were studied to evaluate further the relative contributions of these sub-populations. The T cell receptor expression of the enriched DN T cell population from patients with SLE, RA and healthy controls was examined using three-colour flow cytometry as described in chapter 2, using FITC conjugated antibodies to αβ and γδ TCR. Both αβ TCR^+ and γδ TCR^+ T cells were present in the enriched DN T cell population. Representative histogram profiles showing αβ and γδ TCR expression is shown in figure 3.5.

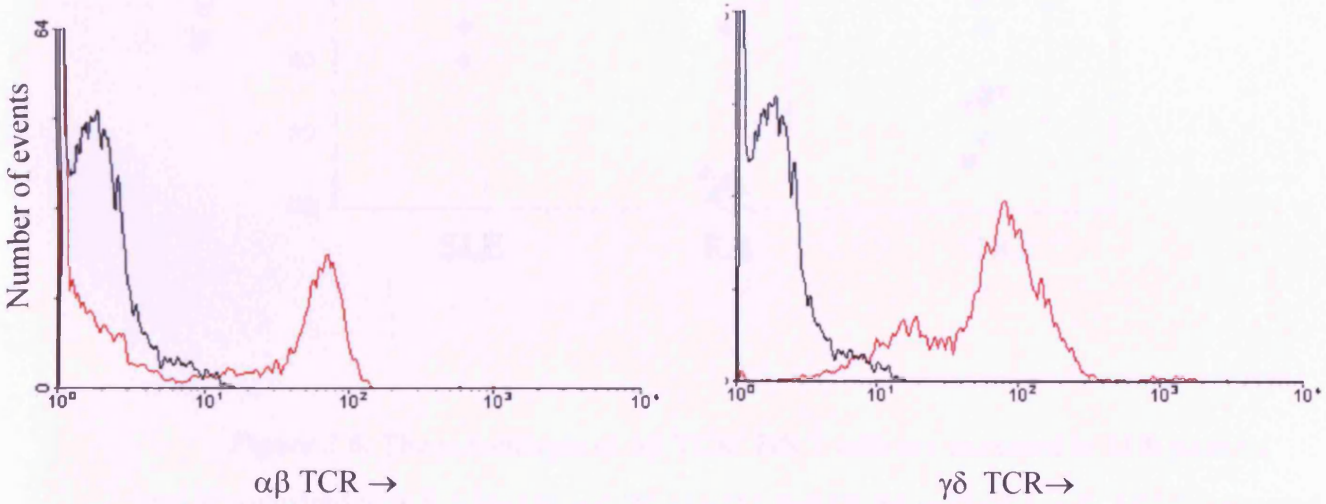


Figure 3.5: Representative histograms showing αβ and γδ TCR expression on DN T cells. Enriched DN T were stained with anti-CD3-PE (FL2), anti-CD4 and anti-CD8 CyChrome (FL3) conjugated and defined as $CD3^+ CD4^- CD8^-$. TCR was studied using anti-αβ TCR-FITC or anti-γδ TCR-FITC antibodies and analyzed by flow cytometry. Measurements were made on gated enriched DN T cells identified by two-colour immunofluorescence staining as shown in Figure 1.

The percentages αβ TCR+ve DN T cells in SLE patients within the total DN population (59.81±10.39) were significantly increased (p=0.04) compared with RA (53.08± 21.91) and HC (48.24±15.23). The results are shown on figure 3.6. Detailed examination of the treatment regimes at the time of sampling showed that while many of the patients were on steroid therapy, either alone or in combination with other drugs, seven were receiving no major drug therapy.

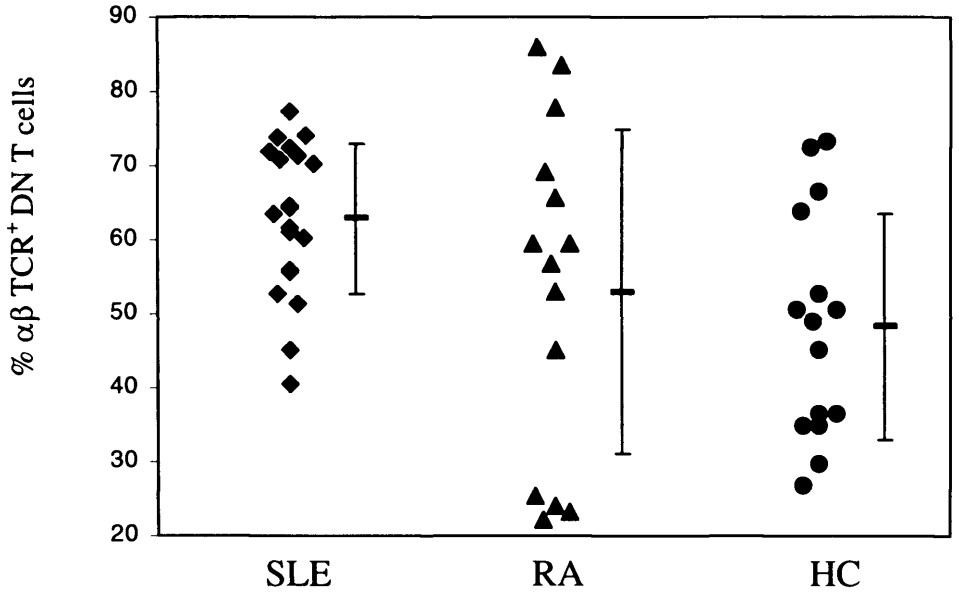


Figure 3.6: The percentages of αβ TCR^+ DN T cells are increased in SLE patients (n=18) compared with RA (n=14) and HC (n=15). p=0.02 for increase in $\alpha\beta^+$ DN T compared with both RA and HC. Enriched DN T cells were triple stained for CD4/CD8 (CyChrome), CD3 (PE) and αβ TCR (FITC). Cells gated on an electronic DN T cell gate ($CD3^+CD4^-CD8^-$) were analyzed for αβ TCR (FITC) expression.

3.2.7: Density of CD3:TCR expression in DN T cells.

To define the distinct characteristics of DN T cells further, the density of expression of CD3: TCR complex on the surface of these cells was examined. The simultaneous staining of PBMC's and enriched DN T cells for CD3 (PE), CD4 (CyChrome), CD8 (CyChrome) and TCR (FITC) permitted qualitative observation of the levels of expression of these markers in different subpopulations. The mean fluorescence intensity (MFI) of CD3 staining on the surface of αβ and γδ TCR expressing DN T cells and CD4/CD8 single positive T cells was evaluated. The results are shown in figure 3.7. As can be seen, αβ TCR^+ DN T cells from patients with SLE, expressed consistently lower levels of CD3 compared to γδ TCR+ve DN T cells (p=0.03) and single positive $CD4^+/CD8^+$ subpopulations (p=0.001). P values were calculated using the Wilcoxon signed rank test. The lower levels of CD3: TCR expression were found to be an intrinsic characteristic of all αβ TCR^+ DN T cells, whether from patients with SLE , RA or healthy individuals(data not shown).

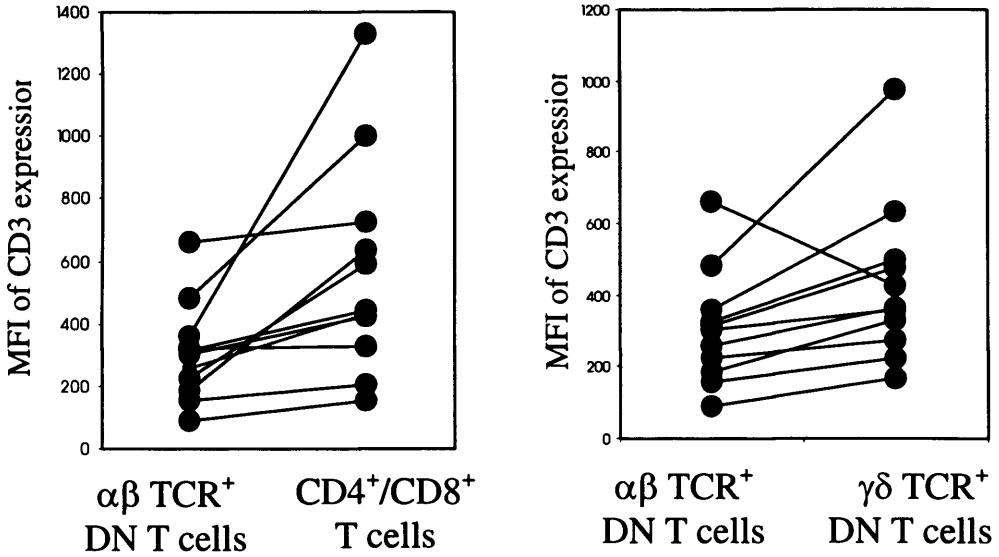


Figure 3.7: Mean Fluorescence Intensity (MFI) of CD3 expression on αβ TCR^+ DN T cells (n=11) from patients with SLE was lower than values for γδ TCR^+ DN T cells (p=0.03) and $CD4^+/CD8^+$ T cells (p=0.001). Enriched DN T cells were triple stained for

CD4/CD8(CyChrome), CD3(PE) and αβ TCR or γδ TCR (FITC). Cells gated on an electronic DN T cell gate ($CD3^+CD4^-CD8^-$) were analyzed for αβ TCR or γδ TCR (FITC) expression.

3.2.8: Levels of $\gamma\delta^+$ TCR^+ T cells remain unchanged patients with SLE when compared to patients with RA and healthy controls but numbers of $\gamma\delta^+$ TCR^+ DN T cells are decreased in patients with SLE .

The percentages of T cells expressing γδ TCR were studied in patients with SLE, RA and healthy controls using three-colour immunofluoresence staining as described in chapter 2. The cell population studied included both DN T cells and T cells expressing CD4 or CD8 surface co-receptors. The results are shown in Figure 3.8. There was no difference in the percentages of γδ T cells in patients with SLE compared to healthy controls (p = 0.12) and patients with RA (p = 0.9).

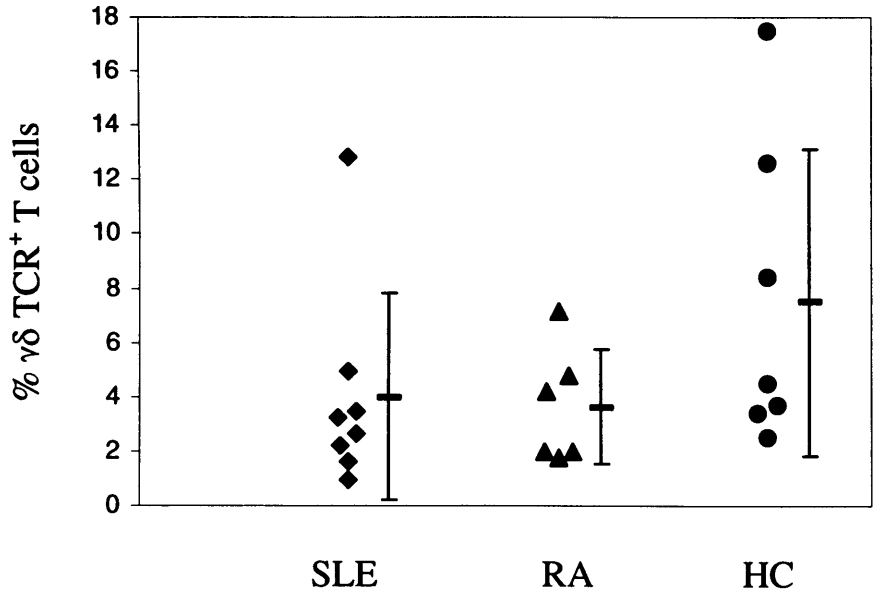


Figure 3.8: Percentages of γδ TCR^+ T cells are similar in patients with SLE (n=8), RA (n=6)(p = 0.9) and HC (n=7) (p = 0.12). Blood PBMCs were triple stained for CD4/CD8(CyChrome), CD3(PE) and γδ TCR (FITC). Cells gated on an electronic Lymphocyte gate were analyzed γδ TCR (FITC) expression.

When the percentage of γδ TCR^+ DN T cells were examined in enriched DN T cell populations, both patients with SLE (p=0.0054)and RA (p=0.016) showed significantly reduced percentages compared with healthy controls (SLE: 44.16±12.72; RA: 40.71±20.81; HC: 58±13.34). These results are shown in Figure 3.9.

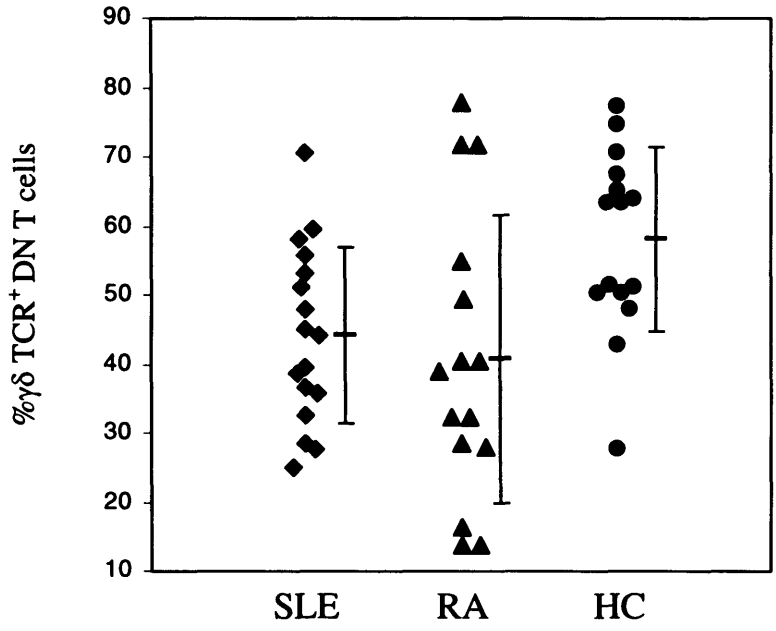


Figure 3.9: Percentages of γδ TCR^+ DN T cells are reduced in patients with SLE (n=17) (p=0.0054), and RA (n=15) (p = 0.0161) when compared to HC (n=15). Enriched DN T cell populations were triple stained for CD4/CD8 (CyChrome), CD3 (PE) and γδ TCR (FITC).Cells gated on an electronic DN T cell ($CD3^+$ $CD4^-$ $CD8^-$) gate were analyzed for γδ TCR (FITC) expression.

3.2.9: Summary of results from chapter 3.

The data from this chapter can be summarized as follows:

i. The percentages of $CD3^+$ T cells in patients with SLE were unchanged when compared to patients with RA or healthy controls ($p = 0.4$)

ii. The percentages of total double negative ($\alpha\beta$ and $\gamma\delta$ TCR^+) T cells was not significantly increased in patients with SLE when compared to RA patients ($p = 0.3$) and healthy controls ($p = 0.3$). The percentages of DN T cells also showed no correlation with the disease activity (global score) in patients with SLE ($p = 0.8$).

iii. There was a significant increase in the $\alpha\beta$ TCR expressing sub-population of DN T cells in patients with SLE compared to RA patients and healthy controls ($p = 0.02$).

iv. The $\alpha\beta$ TCR^+ DN T cells from both patients with SLE and healthy controls displayed significantly lower levels of expression of the CD3: TCR complex on their cell surface than their $\gamma\delta$ TCR^+($p = 0.03$) counterparts and single positive ($CD4^+/8^+$) T cells ($p = 0.001$).

v. The percentage of total $\gamma\delta$ TCR expressing T cells was unchanged between SLE patients, RA patients ($p = 0.9$) and healthy controls ($p = 0.12$). The percentages of $\gamma\delta$ TCR expressing DN T cells however was reduced in patients with SLE ($p=0.0054$) and RA ($p=0.016$) compared to healthy controls.

3.3: Discussion.

3.3.1: T cells and DN T cells in SLE.

As discussed in the introduction, the pathogenesis of SLE has been established as a T cell dependent process prompting a detailed study of the numbers and functions of this group of cells, in patients with SLE (Cohen, 1993). In this study, we did not find increased percentages of T cells in patients with SLE, compared to RA patients and healthy controls.

An increased percentage of $CD3^+$T cells, in patients with SLE, was reported by Erkeller-Yüksel et al, in 1993 (Erkeller-Yusel *et al.*, 1993). In contrast, other studies have found a decrease in T cell percentages in SLE patients when compared to healthy controls (Glinski *et al.*, 1976; Smolen *et al.*, 1982). We examined the percentages of $CD3^+$ T cells in patients with SLE and found them unchanged, when compared to both autoimmune disease controls (RA patients) and healthy individuals. The variable results reported in the numbers of T cells in SLE patients might be because of the larger cohort of patients (71 patients) in some of the studies (Erkeller-Yusel *et al.*, 1993) and the heterogeneous nature of patients included in each of the studies.

I also failed to find an increase in the total DN T cell population ($\alpha\beta$ or $\gamma\delta$ TCR expressing DN T cells) in SLE patients we studied compared with autoimmune controls (RA patients) and healthy subjects. This is in contrast to some previous reports on the total DN T cell population in SLE. These studies have indicated the presence of increased percentages of this population in patients with SLE (Lacki *et al.*, 1997; Devi *et al.*, 1998). These reports also indicated that in patients with SLE who have consistently elevated levels of DN T cells, treatment with corticosteroids and cyclophosphamide reduced the percentages of these cells. Interestingly, although my data does not suggest a statistically significant expansion in the total DN T cell population in SLE patients compared to RA patients and healthy controls, a few patients in the study showed a large increase in their DN T cell population though this was not related to their disease activity. We studied this population in patients with variable disease activity (as indicated by their disease activity global score) and could find no correlation between the percentages of total DN T cells and disease activity. The patient population studied, consisted of both patients receiving immunosuppressive therapy and those receiving no drug treatment.

3.3.2: αβ TCR$^+$ DN T cells in SLE.

On further analysis of the DN T cell populations expressing either the αβ or γδ TCRs, the percentages of αβ TCR$^+$ DN T cells in patients with SLE, within the total DN population were significantly increased compared with RA and HC. It is unlikely that the observed increase was a result of therapy because, although many of the patients were receiving steroids, either alone or in combination with other drugs, eight patients were not receiving any major drugs. There was no significant increase in the numbers of αβ TCR$^+$ DN T cells in patients with RA compared with HC demonstrating that this expanded population is specific to SLE. These data are consistant with other reports of expanded populations of αβ TCR$^+$ DN T cells in patients with autoimmune diseases such as SLE and systemic sclerosis (Shivakumar *et al.*, 1989; Sakamoto *et al.*, 1992; Liu *et al.*, 1998; Sieling *et al.*, 2000). On the other hand, long term clonal proliferation of αβ TCR$^+$ DN T cells was not found to be associated with any history of severe illness in two apparently healthy subjects (Kusunoki *et al.*, 1992).

3.3.3: CD3:TCR complex on αβ TCR+ve DN T cells.

One of the distinctive characteristics of αβ TCR$^+$ DN T cells previously reported, is the expression of lower levels of the CD3: TCR complex on the cell surface. My data show that the density of expression of both CD3 and the αβ TCR (as indicated by the MFI of anti-CD3 or TCR αβ surface staining) was found to be reduced significantly in SLE DN T cells, when compared to CD4$^+$ or CD8$^+$ T cells. Interestingly this decreased expression of CD3 was not peculiar to SLE since it was found in patients with RA, healthy individuals and has been previously reported in, patients with adult T cell leukemia (ATL), systemic sclerosis and in murine DN T cells (Murison *et al.*, 1993; Sakamoto *et al.*, 1992; Suzushima *et al.*, 1993; Huang and Crispe, 1992). My data also suggests a significant reduction in CD3 expression on all αβ TCR$^+$ DN T cells compared with γδ TCR$^+$ DN T cells. This characteristic was also common to DN T cells from patients with SLE, RA and healthy individuals. Surprisingly, the CD3: TCR complex has been shown to be functionally competent in αβ TCR$^+$ DN T cells, so the functional significance of the paucity of CD3: TCR expression is unclear and requires further study (Groh *et al.*, 1989; Rivas *et al.*, 1990; Matsumoto *et al.*, 1991).

3.3.4: γδ T cells in SLE.

No change in the percentage of total TCR γδ expressing T cells (both DN and $CD8^+$) in patients with SLE compared to RA or HC was seen in this study. This observation, however does not rule out a role for this population as the γδ T cells in the particular patients included in our study might have been 'trafficked away' to the sites of local inflammation in various organ systems. The role of γδ T cells in patients with SLE is the subject of many contradicting reports in literature. Although some investigators suggest an expansion of this population in the peripheral blood of patients with SLE, others report unchanged or decreased numbers in the peripheral blood (Gerli *et al.*, 1991;Riccieri *et al.*, 2000 ; Robak *et al.*, 2001 ; Robak *et al.*, 1999).

3.4: Concluding remarks.

An expanded population of αβ TCR^+ DN T cells relative to other T cells in SLE patients was found although this was not directly related to their disease activity. The next question is related to whether DN T cells in SLE patients are 'activated' suggesting that they could be functionally important in the disease.

Chapter 4.

Activation status of DN T cells in patients with SLE.

4.1: Introduction and aims.

4.1.1: Introduction.

4.1.1.1: Expression of activation markers by T cells in SLE patients.

For conventional T cells, activation is initiated by interaction of the CD3: TCR complex with a processed antigenic peptide bound to class I or class II MHC molecule on the surface of antigen-presenting cell. This interaction and the resulting activating signals also involve a variety of accessory molecules on the T cell and antigen-presenting cell (APC).

Following the interaction of T cell with antigen, numerous genes are activated. The gene-products can be grouped into categories depending on how early they can be detected following activation:

Early genes: expressed within 1-2 hours of antigen recognition; encode IL-2, IL-2R, IL-3, IFN-γ, IL-6, CD69 and numerous other proteins.

Late genes: expressed more than 2 days after antigen recognition; encode various adhesion molecules, HLA-DR etc (Kuby, 1997).

Thus characterising the activation phenotype of T cells can provide clues to their participation in on-going immune reactions. The activation of DN T cells is thought to be through unconventional antigen presenting molecules like CD1, but is still believed to be accompanied by the expression of activation molecules expressed by conventional T cells. Therefore, in order to understand their potential role in the pathogenesis of SLE, we have evaluated the expression of a number of these activation molecules in the DN T cell population of patients with SLE.

A number of studies on the expression of early and late activation markers on SLE lymphocytes have been reported to date. The most commonly studied activation markers are CD69, HLA-DR, CD45 and the CD28/CTLA-4 co-stimulatory molecules.

4.1.1.2: CD69.

CD69 is a phosphorylated disulfide-linked homodimer that appears on the surface of human T, B cells and thymocytes in the early steps of activation; its molecular mass is 28 to 34 kDa under reducing conditions. This molecule is able to mediate positive signals to the lymphocytes as the anti-CD69 mAb (MLR3, AIM, Leu 23) in synergism with phorbol esters, induce IL-2 production and proliferation of lymphocytes. The relevance of CD69 in the activation process is also suggested by the broad range of signals able to modulate CD69 on T cells. In fact, not only the mitogens or the CD3-promoted activation, but also the alternative pathways mediated by CD2 or CD28 are accompanied by CD69 expression; moreover a very rapid and transient appearance of CD69 on the cell surface is observed also in response to a stimulus not specifically involved in T cell activation such as heat shock (Barclay, 1997). Crosslinking of CD69 generates intracellular signals in all cell lineages studied, both mouse and human, and results in a variety of cellular end responses such as cell proliferation and cytokine secretion. Since a specific ligand has not yet been identified, a definite functional identity for CD69 is still missing. However, the broad expression of CD69 and its conserved ability to generate intracellular signals suggests a general role for the CD69 receptor in the biology of hematopoietic cells (Marzio *et al.*, 1999).

Some studies have reported increased constitutive CD69 expression on T cells in murine models of lupus and human SLE (Sobel *et al.*, 1999; Ishikawa *et al.*, 1998; Portales-Perez *et al.*, 1997) (Crispin *et al.*, 1998). Other studies on larger numbers (n=30) of patients do not, however, confirm these results (Fernandez-Gutierrez *et al.*, 1998; Afeltra *et al.*, 1993). Abnormalities in CD69 expression after activation of T cells from patients with SLE with anti-CD3, anti-CD2 monoclonal antibodies or mitogens such as phytohaemagglutinin (PHA), phorbol myristate acetate (PMA) have been reported by several investigators (Fernandez-Gutierrez *et al.*, 1998; Portales-Perez *et al.*, 1997; Crispin *et al.*, 1998). Increased numbers of CD69 bearing cells and their hypo-responsiveness to stimulation in culture have also been reported in synovial fluid but not peripheral blood T cells of patients with RA (Afeltra *et al.*, 1993; Hernandez-Garcia *et al.*, 1996).

Su et al, report a high correlation between CD69/CD3 ratios and SLEDAI scores of disease activity in patients with SLE, and suggest it could be used as an estimation of disease activity when levels of anti-dsDNA, C3 and C4 fail to show good correlation (Su *et al.*, 1997).

4.1.1.3: HLA-DR.

HLA-DR, a MHC class II molecule is expressed on dendritic cells, B cells, monocytes, macrophages, myeloid and erythroid precursors and some epithelial cells. MHC-class II is also expressed on activated T cells in humans and rats, where expression is regulated by cytokines including interferon-γ (Seddon and Mason, 1996). MHC class II molecules present exogenously derived antigen to $CD4^+$ T lymphocytes, which are usually helper T cells (Rudensky, 1995).

Previous studies had reported a higher proportion of HLA-DR^+ T cells in both the $CD4^+$ and $CD8^+$ T cells in patients with SLE which correlated with disease activity.(Tokano *et al.*, 1997; Spronk *et al.*, 1996) (Hashimoto *et al.*, 1994; Bijl *et al.*, 2001).

4.1.1.4: CD28/B7 system of T cell costimulation.

T cell costimulatory interactions are necessary for effective lymphocyte activation and and also serve to enhance the immune response. It is now accepted that a major costimulatory pathway involves the CD28 molecule. CD28 interactions with the B7 family of costimulatory ligands are essential for initiating antigen-specific T cell responses, up-regulating cytokine expression and promoting T cell expansion and differentiation (June *et al.*, 1994). The CD28 glycoprotein is expressed constitutively on the surface of 80% of human T cells. CD28 expression is not static because the levels of CD28 increase on T cells following activation, but ligation of CD28 leads to transient downregulation. Cytotoxic T lymphocyte antigen- 4 (CTLA-4), the other component of the CD28/B7 T cell costimulatory system is not constitutively expressed on T cells. Instead, it is upregulated following T cell activation due to positive regulatory elements(Lenschow *et al.*, 1996). In both human and murine systems, CTLA-4 expression peaks during period of transient downregulation and less responsiveness of CD28, suggesting that CTLA-4 might be functionally active at a time when CD28 function is impaired (Allison and Krummel, 1995). The function of CTLA-4 remains controversial and it is suggested that during T cell activation, CTLA-4 is upregulated and through interactions with its ligands (B7) may facilitate or promote downregulation of the immune response (Allison and Krummel, 1995; Lenschow *et al.*, 1996). It has been reported that patients with

SLE have a preferential decrease of $CD28^+$ T cells, especially in the $CD8^+$ T cell compartment (Kaneko *et al.*, 1996; Garcia-Cozar *et al.*, 1996; Horwitz *et al.*, 1997; Kaneko *et al.*, 1996).

It has been suggested that the absence of costimulators on normal tissue cells could serve to induce self-tolerance and that inappropriate expression of costimulators on antigen-presenting cells (APC) could activate self-reactive T cells, resulting in autoimmunity (Kaneko *et al.*, 1997).

Based on function and experimental data, the gene encoding cytotoxic T lymphocyte-associated antigen 4 (CTLA4) has been suggested as a candidate gene for conferring susceptibility to autoimmunity including SLE (Kristiansen *et al.*, 2000; Pullmann *et al.*, 1999; Heward and Gough, 1997). Patients with SLE have been shown to have increased numbers of $CTLA\text{-}4^+$ T cells compared to controls. In addition, induction of CTLA-4 expression after stimulation with PMA *in vitro* was found to be weaker in T cells from patients with SLE compared to HC (Liu *et al.*, 1998). Further evidence of CD28/CTLA-4-B7 involvement in SLE disease pathogenesis comes from studies where, treatment with soluble CTLA-4 IgG resulted in blocked autoantibody production and prolonged life in murine models of lupus (Finck *et al.*, 1994; Takiguchi *et al.*, 2000).

4.1.1.5: CD45.

CD45 proteins are found on all cells of haematopoietic origin, except erythrocytes and are necessary for signalling through the T cell receptor (Thomas, 1989). Various isoforms of CD45 (CD45 RA, CD45RB, CD45RC and CD45R0) are generated by alternative splicing and are expressed differentially on leucocytes. This differential expression has been of use in defining subsets of T cells, in which naïve T cells express CD45RA whereas most memory cells express CD45R0 (Barclay N, 1997). The associations between expression of various CD45 isoforms on the peripheral blood T ($CD4^+/CD8^+$) lymphocytes, with autoimmune processes and haematological manifestations in SLE have been studied by several investigators. In SLE patients the pattern of CD45 isoform expression (from CD45RA to CD45R0) was found to vary directly with time since the onset of disease symptoms with higher numbers of CD45R0 cells in patients with active disease (Neidhart *et al.*, 1996; Gordon *et al.*, 1996; Hernandez-Fuentes *et al.*, 1999).

4.1.1.6: In vitro T cell activation in SLE.

Previous studies have demonstrated that various T and B cell functions, including immunoglobulin and IL-2 secretion, IL-2R expression, and proliferation of T and B cells in response to antigenic or mitogenic stimulation, are all moderately to severely impaired in SLE (Cohen, 1993). I wished to understand if this hyporesponsiveness was also true of the DN T cell population in patients with SLE or was restricted to the conventional T cell subsets reported in previous studies (Alcocer-Varela *et al.*, 1991; Crispin *et al.*, 1998) (Portales-Perez *et al.*, 1997) (Horwitz *et al.*, 1997; Hernandez-Fuentes *et al.*, 1999).

4.1.2: Aims.

This part of my work aimed to characterize and compare the activation status of DN T cells in patients with SLE, RA and healthy individuals. I have evaluated the expression of early (CD69) and late (HLA-DR) activation markers, the proportion of naïve and memory cells and the status of the CD28/CTLA-4-B7 costimulatory system. Activation of DN T cells from patients with SLE and HC has also been studied in cell cultures stimulated with a mitogen (PHA) for 24 hours.

4.2: Results.

4.2.1: Patients used in this study.

Twenty two patients with SLE (21 females and 1 male aged 17 to 67 years, mean 42 years) were studied with informed consent. Each met four or more of the revised criteria of the American College of Rheumatology for the classification of the disease (Tan *et al.*, 1982). Disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) computerised index (Hay *et al.*, 1993). In this study patients with a global scoring >6 were deemed active ≤6 as inactive. Levels of circulating anti-dsDNA antibodies and C3 were measured during routine patient assessment. Serum levels of anti ds-DNA antibodies in excess of 50 I.U. ml^{-1} (Shield Diagnostics, Dundee) and levels of C3 less than 0.9 I.U. ml^{-1} (by laser nephelometry) are regarded as abnormal. For statistical analysis in this study, anti-dsDNA antibody levels of ≥100 I.U. ml^{-1} or more were considered to be high. Detailed examination of

the treatment regimes at the time of sampling showed that while many of the patients were on steroid therapy, either alone or in combination with other drugs, eight were receiving no major drug therapy (Table 4.1).

Seventeen patients with rheumatoid arthritis (RA: 15 female, 3 male, aged 35 to 82 years, mean 59 years) who fulfilled four or more of the ARA criteria for rheumatoid arthritis and 18 healthy controls (HC: 17 females, 1 male aged 18 to 45 years, mean 31 years) were also studied (Arnett *et al.*, 1988).

Patient No	Global Score	Drug Treatment
19	10	No major drugs
5	3	No major drugs
20	3	No major drugs
8	4	No major drugs
16	5	No major drugs
10	6	No major drugs
21	6	No major drugs
1	4	No major drugs
22	11	No major drugs
18	9	5 mg Prednisolone
9	10	400 mg Hydroxychloroquine
13	0	200mg Plaquanil
6	2	2 mg Prednisolone
		200mg Hydroxychloroquine
17	1	2.5 mg Prednisolone
		400 mg Plaquanil
4	2	5 mg Prednisolone
		400 mg Hydroxychloroquine
7	4	8 mg Prednisolone
		400 mg Hydroxychloroquine
		750 mg quarterly Cyclophosphamide
		10 mg Prednisolone
2	8	400 mg Hydroxychloroquine
		7.5 mg prednisolone
12	7	25 mg Azathioprine
		7.5 mg prednisolone
15	4	150 mg Azathioprine
		400 mg Hydroxychloroquine
11	3	125 mg monthly Deltastab pulse
		50 mg Azathioprine
3	9	125 mg monthly Deltastab pulse

Table 4.1: Disease activity (global score) and treatments of patients with SLE studied in this chapter.

4.2.2: Expression of surface markers on DN T cells of patients with SLE.

The expression of cell surface markers HLA-DR, CD69, CD28, CTLA-4 and CD45RA/R0 in the $CD4^+/CD8^+$ and DN T cell populations of patients with SLE, RA and healthy individuals were examined.

HLA-DR: The percentages of HLA-DR^+ DN T cells were increased in patients with SLE compared to both RA patients (p=0.016) and HC (p=0.01:Fig 4.1a). In addition, there were significantly more HLA-DR^+ DN T cells than CD4/8+ T cells in patients with SLE (p=0.006: Fig 4.1b). However, there was neither any significant difference in percentages of HLA-DR^+ DN T cells nor of $CD4/8^+$ T cells in patients with active disease compared with inactive disease (BILAG Global Disease Activity Index). There was no correlation between the global score and percentage of HLA-DR^+ $CD4/8^+$ T cells (r=0.21, p=0.48) or HLA-DR^+ DN T cells (r=-0.08, p=0.78). With regard to anti dsDNA antibody levels, there were no significant differences in percentages of HLA-DR^+ $CD4/8^+$ T cells or HLA-DR^+ DN T cells between patients with high versus low levels and no correlation between levels of anti dsDNA antibody and percentage of HLA-$DR^+CD4/8^+$ T cells (r=0.35, p=0.25) or HLA-DR^+ DN T cells (r=-0.38, p=0.19). However, when high and low levels of circulating C3 were related to HLA-DR expression, HLA-DR expression was significantly higher in DN T cells from patients with high C3 (p=0.03). There was a positive correlation between C3 level and HLA-DR expression in both CD4/8+ T cells and DN T cells (r=0.57, p=0.04 and r=0.58, p=0.04 respectively). These results are summarised in table 4.2.

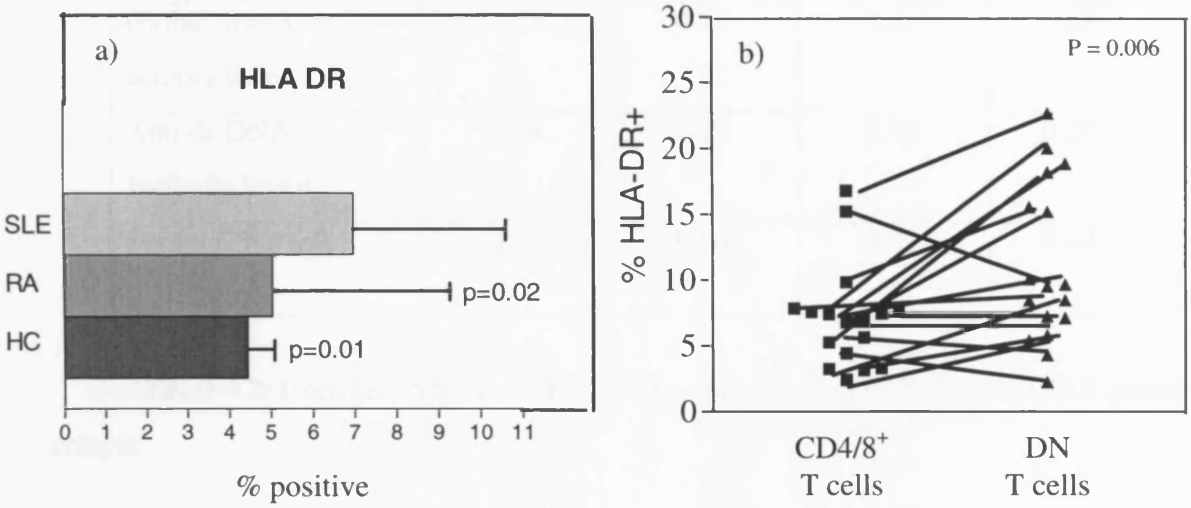


Figure 4.1:

a) Increased percentages of HLA-DR$^+$ DN T cells in patients with SLE (n=17), compared to RA (n=14)(p=0.02) and HC (n=14) (p=0.01).

b) DN T cells in patients with SLE (n=17) show a higher percentage of HLA-DR$^+$ cells compared to the conventional (CD4/8$^+$) population (p=0.006).

Enriched DN T cells were stained with fluorochrome conjugated antibodies to CD3 (PE) (FL2), CD4/CD8 (CyChrome) (FL3) and HLA-DR (FITC) (FL1) and analyzed by flow cytometry. An electronic gate was placed around DN T cells (CD3$^+$ CD4$^-$ CD8$^-$) or single positive (SP) T cells (CD3$^+$, CD4$^+$ or CD8$^+$) and gated cells analyzed for HLA-DR (FL1) expression.

	HLA-DR$^+$ DN T cells		HLA-DR$^+$CD4$^+$/CD8$^+$ T cells	
	r value	p value	r value	p value
Global disease activity index	- 0.08	0.78	0.21	0.48
Anti ds DNA antibody levels	0.38	0.19	0.35	0.25
Serum C3 levels	0.58	0.04	0.57	0.04

Table 4.2: Correlation between HLA-DR expression in DN T cells and SLE patient criteria.

CD69: Although patients with SLE showed an increased percentage of DN T cells expressing CD69 compared to HC, this was not significant (p = 0.08: Fig 4.2a). However, there was a significant increase in CD69$^+$ DN T cells in SLE compared with RA patients (p=0.005). In addition, in the case of RA there was a significantly lower percentage of CD69$^+$ DN T cells compared with the CD69$^+$ CD4/8$^+$ T cell population (p=0.004, data not shown). In patients with SLE, the percentages of DN CD69$^+$ cells were not significantly different from the percentages of CD4/8$^+$CD69$^+$ T cells (Fig 4.2b).

There was no significant difference in CD69$^+$ DN T cells or CD69$^+$ CD4/8$^+$ T cells in those patients with active disease compared with inactive disease. In addition there was no correlation between either CD69$^+$ DN or CD69$^+$ CD4/8$^+$ expression and BILAG Global Disease Activity Index (r=0.21, p=0.54 and r=-0.55-0.52, p=0.95 respectively). No significant differences in percentages of CD69$^+$ DN or CD69$^+$ CD4/8$^+$ were seen between high and low levels of serum anti dsDNA and no correlation was found between percentage of CD69$^+$ CD4/8$^+$ T cells (r=0.05, p=0.87) or CD69$^+$ DN T cells (r=0.33, p=0.31) and level of anti dsDNA antibody. In addition, no significant differences were seen between low and high levels of serum C3 and percentage of CD69$^+$ DN or CD69$^+$ CD4/8$^+$ and no correlation between percentage of either CD69$^+$ DN (r=0.49, p=0.12) or CD69$^+$ CD4/8$^+$ (r=-0.38, p=0.25) and level of anti dsDNA antibody. These results are summarised in table 4.3.

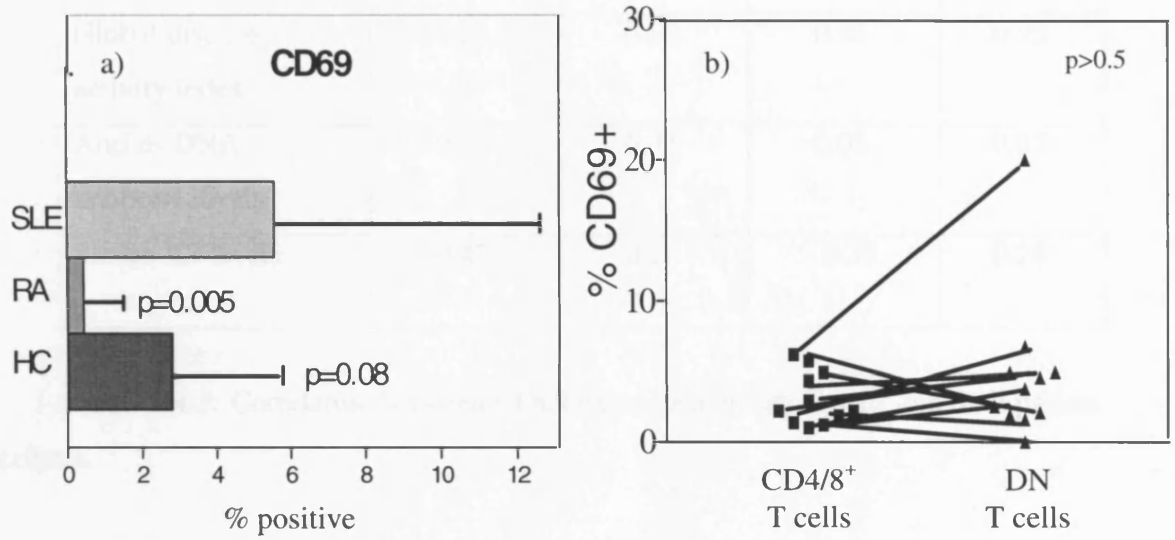


Figure 4.2:

a) CD69 expression is significantly increased in DN T cells from patients with SLE (n=11) compared to patients with RA (n=11)(p=0.005). In HC (n=9), expression of CD69 is lower but the difference is not significant.

b) There is no significant difference in expression of CD69 between $CD4/8^+$ T cells and DN T cells(n=11) in patients with SLE.

Enriched DN T cells were stained with fluorochrome conjugated antibodies to CD3 (PE) (FL2), CD4/CD8 (CyChrome) (FL3) and CD69 (FITC) (FL1) and analyzed by flow cytometry. An electronic gate was placed around DN T cells ($CD3^+$ $CD4^-$ $CD8^-$) or single positive (SP) T cells ($CD3^+$, $CD4^+$ or $CD8^+$) and gated cells analyzed for CD69 (FL1) expression.

	$CD69^+$ DN T cells		$CD69^+CD4^+/CD8^+$ T cells	
	r value	p value	r value	p value
Global disease activity index	0.21	0.54	0.55	0.95
Anti ds DNA antibody levels	0.33	0.31	0.05	0.87
Serum C3 levels	0.49	0.12	- 0.38	0.25

Table 4.3: Correlation between CD69 expression in DN T cells and SLE patient criteria.

CD28: There were more DN T cells expressing the costimulatory molecule CD28 in SLE patients than in either RA (p= <0.0001) or HC (p=0.002: Fig 4.3a): Higher levels of $CD28^+$ DN T cells were seen compared with $CD28^+CD4^+/CD8^+$ T cells populations in SLE (p=0.05: Fig 4.3b).

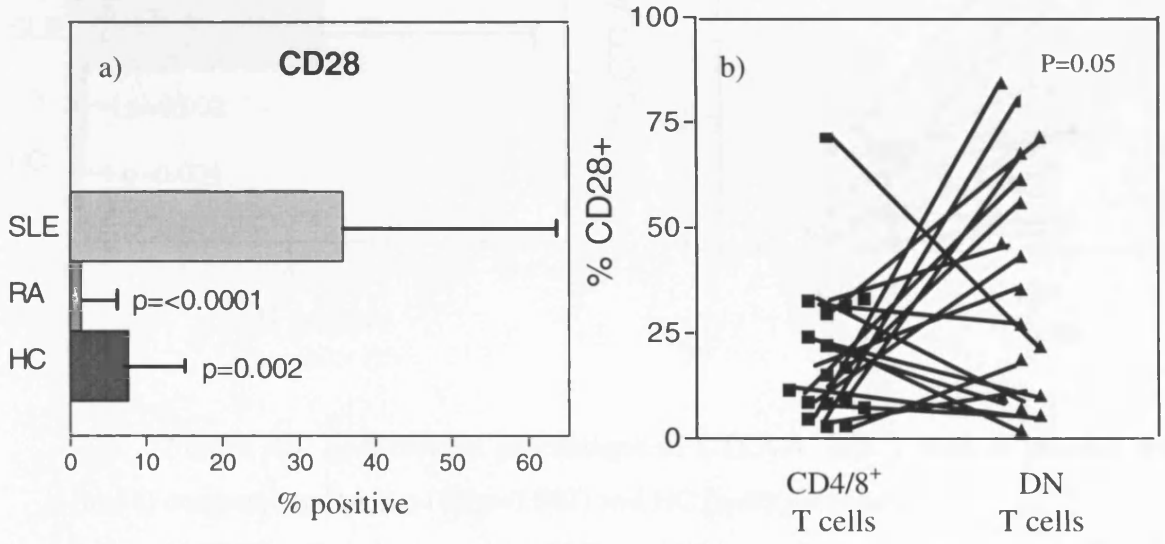


Figure 4.3: a) Increased percentages of $CD28^+$ DN T cells in patients with SLE (n=17) compared to RA (n=15)(p<0.0001) and HC (n=12)(p=0.002).

b) DN T cells in patients with SLE (n=17) show a higher percentage of $CD28^+$ cells compared to the conventional ($CD4/8^+$) population (p=0.05).

Enriched DN T cells were stained with fluorochrome conjugated antibodies to CD3 (PE) (FL2), CD4/CD8 (CyChrome) (FL3) and unconjugated anti-CD28 antibody, followed by FITC-conjugated secondary antibody (FL1) and analyzed by flow cytometry. An electronic gate was placed around DN T cells ($CD3^+$ $CD4^-$ $CD8^-$) or single positive (SP) T cells ($CD3^+$, $CD4^+$ or $CD8^+$) and gated cells analyzed for CD28 (FL1) expression.

CTLA-4: Although few $CTLA\text{-}4^+$ cells were detected (Fig 4.4a), there were significantly more in the DN T cell population of the patients with SLE compared with both the RA patients (p= 0.002) and HC (p= 0.004). Numbers of $CTLA\text{-}4^+$ $CD4/8^+$ T cells also appeared to be increased compared to control groups but the increase was not statistically significant. The percentages of $CTLA\text{-}4^+$ DN T cells were significantly higher than the $CTLA\text{-}4^+CD4^+/CD8^+$ cells in both the SLE patients (p=0.03: Fig 4.4b) and HC (p=0.001:data not shown).

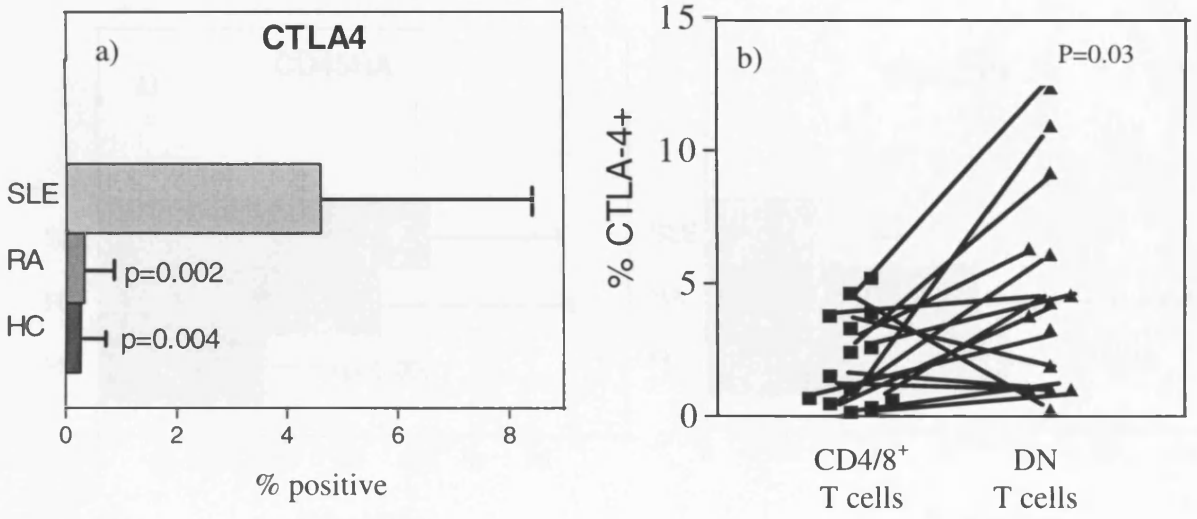


Figure 4.4: a) Increased percentages of $CTLA\text{-}4^+$ DN T cells in patients with SLE (n=14) compared to RA (n=10)(p=0.002) and HC (n=8)(p=0.004).

b) DN T cells in patients with SLE (n=14) show a higher percentage of $CTLA\text{-}4^+$ cells compared to the conventional ($CD4/8^+$) population (p=0.003).

Enriched DN T cells were stained with fluorochrome conjugated antibodies to CD3 (PE) (FL2), CD4/CD8 (CyChrome) (FL3) and CTLA-4 (FITC) (FL1) and analyzed by flow cytometry. An electronic gate was placed around DN T cells ($CD3^+$ $CD4^-$ $CD8^-$) or single

positive (SP) T cells ($CD3^+$, $CD4^+$ or $CD8^+$) and gated cells analyzed for CTLA-4 (FL1) expression.

***CD45RA/RO*:** DN T cells from patients with SLE showed significantly increased percentages of $CD45RA^+$ compared with HC ($p = 0.007$) but not with RA controls (Fig 4.5a). Conversely, SLE patients had significantly lower percentages of $CD45RO^+$ T cells in comparison with HC ($p=0.03$) but not with RA controls (Fig 4.5b). In patients with SLE, the DN population was significantly increased in $CD45RA^+$ compartment compared with the $CD4/CD8^+$ population ($p=0.048$: Fig 4. 6a) while it was significantly decreased in CD45R0 ($p=0.03$: Fig 4.6b).

There were insufficient data to determine accurately whether there was any correlation between disease activity, anti dsDNA or C3 and the levels of expression of CD28, CTLA4, CD45RA or CD45RO.

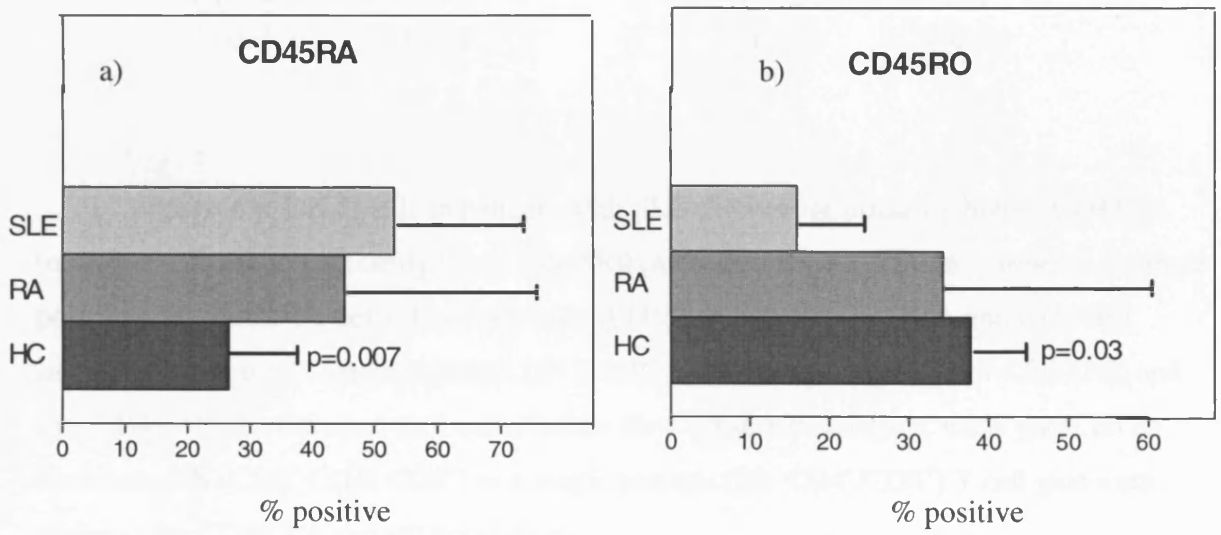


Figure 4.5: DN T cells from patients with SLE (n=15) showed significantly increased percentages of $CD45RA^+$ cells ($p=0.007$) and decreased percentages of $CD45R0^+$($p=0.03$) cells compared to patients with HC (n=9). There were no significant differences in the percentages of $CD45RA^+$ or $CD45R0^+$ DN T cells between patients with SLE and RA(n=12). Fluorochrome (FITC) conjugated CD45RA and CD45R0 antibodies were used to stain enriched DN T cells alongwith anti-CD3 (PE) and anti-CD4/CD8 (CyChrome) antibodies

before flow cytometric analysis. Cells gated on an electronic DN T cell gate ($CD3^+$ $CD4^+$ $CD8^+$) were analyzed for CD45 RA and R0 expression.

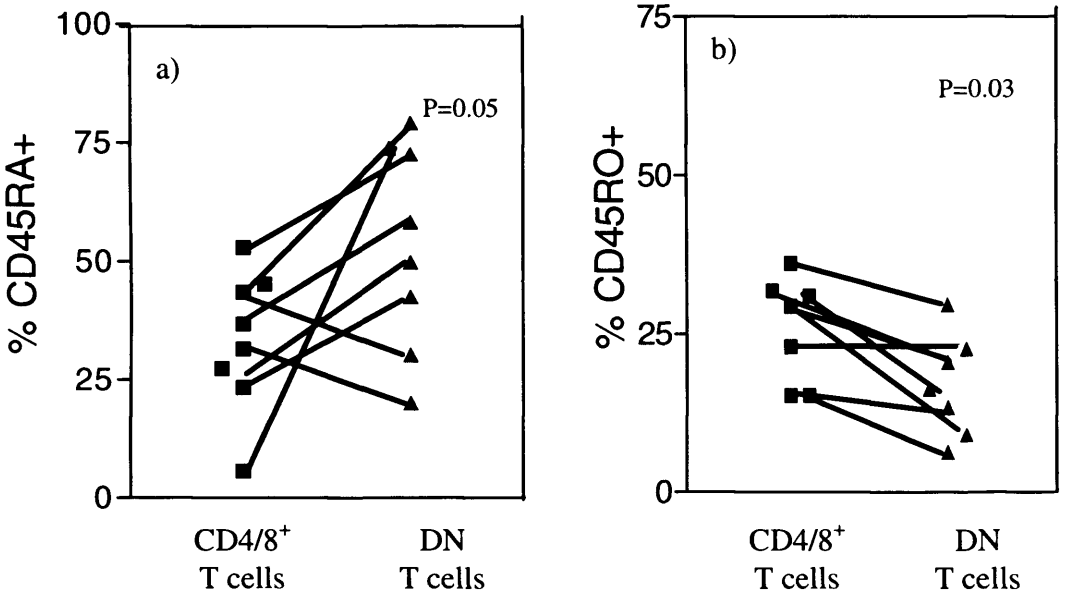


Figure 4.6: DN T cells in patients with SLE showed significantly higher CD45RA (n=8)(p=0.05) and significantly lower CD45R0 (n=7)(p=0.03) expression compared to single positive ($CD4/CD8^+$) T cells. Fluorochrome (FITC) conjugated CD45RA and CD45R0 antibodies were used to stain enriched DN T cells and T cells alongwith anti-CD3 (PE) and anti-CD4/CD8 (CyChrome) antibodies before flow cytometric analysis. Cells gated on an electronic DN ($CD3^+$ $CD4^+$ $CD8^+$) or a single positive (SP: $CD4^+/CD8^+$) T cell gate were analyzed for CD45 RA and R0 expression.

4.2.3: DN T cells from patients with SLE are hypo-responsive to stimulation with Phytohaemaglutinin (PHA) in in-vitro cultures.

To study the effect of mitogen stimulation on DN T cells from patients and healthy controls, we evaluated the expression of HLA-DR on enriched DN T cell cultures, 24 hours after addition of PHA. PHA as a stimulant was chosen because it is a potent and specific T cell

activator. HLA-DR was chosen as an activation marker in these studies as it is a late activation marker appearing 24-48 hours after activation.

The number of DN T cells from patients with SLE expressing HLA-DR after PHA stimulation was significantly lower as shown in figure 4.7.

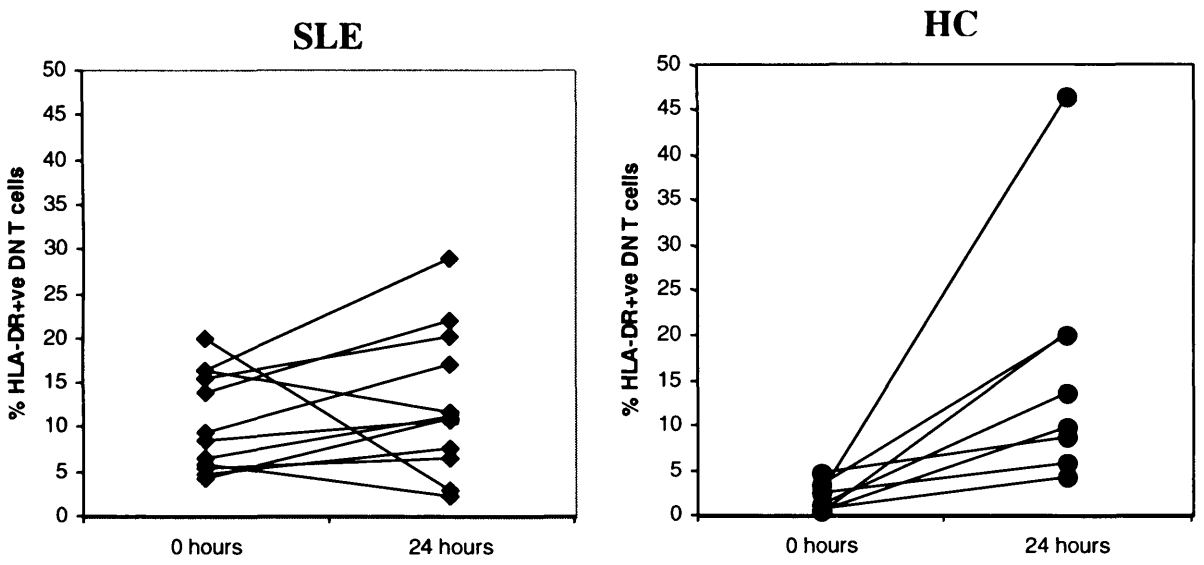


Figure 4.7: Percentages of HLA-DR$^+$ DN T cells from patients with SLE (n=12) and HC (n=8) 24 hours after stimulation with PHA. Enriched DN T cells were stimulated with PHA for 24 hrs in *in vitro* cultures and HLA-DR (FITC) expression analysed by flow cytometric staining. Antibodies to CD3 (PE), CD4 and CD8 (CyChrome) were used to identify and place electronic gates on the DN T cell compartment.

The percentage increase in the number of activated (HLA-DR$^+$) DN T cells over 24 hours after PHA stimulation was also calculated. DN T cells from patients with SLE were hyporesponsive to PHA stimulation, and showed a significantly lower percentage increase in numbers of activated cells compared to healthy controls ($P<0.0001$)(Mann-Whitney U-test). These results are shown in figure 4.8.

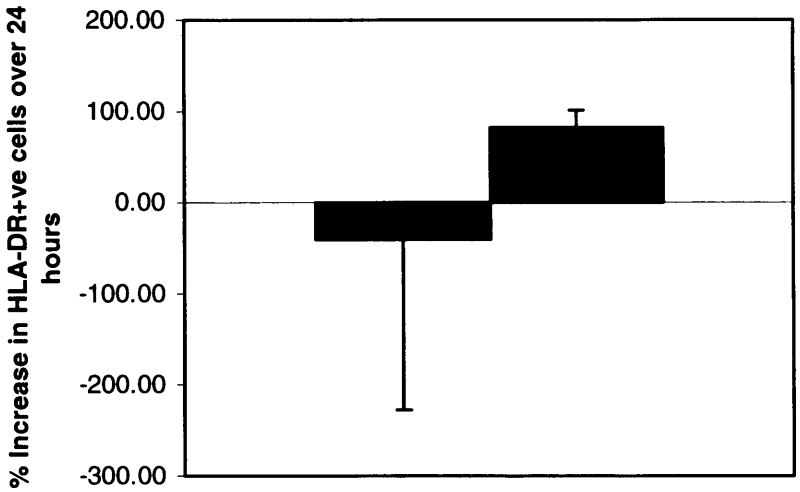


Figure 4.8: Percentage increase in HLA-DR$^+$ DN T cells from patients with SLE (grey) and HC (black) in culture, 24 hours after PHA stimulation. Data shows Mean± standard deviation. Percentage increase was calculated using the equation:

$$\frac{(\%\text{HLA-DR}^+\text{DN T cells at t = 24hrs}) - (\%\text{HLA-DR}^+\text{DN T cells at t = 0hrs})}{(\%\text{HLA-DR}^+\text{DN T cells at t = 24hrs})} \times 100$$

4.2.4: Summary of results from chapter 4.

The data from this chapter can be summarized as follows:

i. Increased numbers of freshly isolated DN T cells from patients with SLE were HLA-DR$^+$ when compared to RA(p=0.016) and HC (p=0.01). The numbers of HLA-DR$^+$ DN T cells was significantly greater than the number of HLA-DR$^+$ CD4$^+$/CD8$^+$ T cells, in the peripheral blood of patients with SLE (p=0.006).

ii. There was a slight but non-significant, increase in the number of DN T cells from patients expressing CD69, in the peripheral blood of SLE patients compared to patients with RA and HC (p=0.08).

iii. The numbers of CD28$^+$ DN T cells in the peripheral blood of SLE patients was significantly higher than in patients with RA (p=<0.0001) or HC (p=0.002). In addition, the numbers of CD28$^+$ DN T cells was significantly greater than CD28$^+$ CD4$^+$/CD8$^+$ T cells in the peripheral blood of patients with SLE(p=0.05).

iv. The numbers of CTLA-4$^+$ DN T cells in the peripheral blood of SLE patients were significantly higher than in patients with RA (p=0.002) or HC (p=0.004). In freshly isolated lymphocytes, the numbers of CTLA-4$^+$ DN T cells were significantly greater than CTLA-4$^+$ CD4$^+$/CD8$^+$ T cells, in patients with SLE (p=0.03).

v. Patients with SLE, showed a higher proportion of "naïve"(CD45RA$^+$) to "memory" (CD45R0$^+$) DN T cells, compared both to their conventional T cell compartment (CD4$^+$/CD8$^+$) (CD45RA- p=0.05, CD45R0-p=0.03) and the DN T cells of control subjects (HC) (CD45RA-p=0.007, CD45R0-p=0.03).

4.3: Discussion.

4.3.1: Activation status of SLE DN T cells.

The data in this chapter indicates that DN T cells in patients with SLE have an activated phenotype. The expression of activation markers by the DN Tcells of patients could indicate that they are functionally important in the disease.

4.3.1.1: HLA-DR.

Increased percentages of DN T cells expressing HLA-DR in patients with SLE compared with the two control groups (RA and HC) were found. HLA-DR is mainly found expressed by T cells. However, there was a high level of HLA-DR^+ cells in the DN T cell compartment compared with the $CD4^+$/ $CD8^+$ T cells in SLE. This finding suggested that the DN T cells are more activated and is further evidence that they could be playing a role in SLE disease pathogenesis. No significant differences in HLA-DR expression between patients with SLE and the control groups (RA and HC) in the conventional $CD4^+/CD8^+$ population were found. Several studies have reported increased numbers of HLA-DR^+ T lymphocytes in peripheral blood and tissues of SLE patients with active disease (Groen *et al.*, 1993; Hashimoto *et al.*, 1994; Heiligenhaus *et al.*, 1996; Tokano *et al.*, 1997). Surprisingly, patients with inactive or clinically quiescent disease also showed large numbers of HLA-DR^+ lymphocytes (Spronk *et al.*, 1996; Spronk *et al.*, 1993). The persistent presence of a high proportion of activated T cells in both patients with active or quiescent disease is of interest with respect to our understanding of the possible pathogenesis of this condition. Although many factors, including viral agents have been proposed, the factors that sustain immunoactivation even during quiescent lupus are unknown (Blomberg *et al.*, 1994; Fredriksen *et al.*, 1993; Hardgrave *et al.*, 1993). It is unclear why there were no differences in expression of the overall $CD4^+/CD8^+$ cells and unlikely that these findings were due to the disease activity of our patient cohort, as there does not appear to be any correlation between BILAG Global Disease Activity Index and HLA-DR expression.

However, there was a positive correlation between C3 levels and HLA-DR expression in both $CD4^+/CD8^+$ T cells and DN T cells. Higher levels of circulating C3 are indicative of less active disease and this finding might indicate that highly activated DN T cells could have

a protective effect. Studies have reported no relationship between deficient PKA-I activity and the proportion of HLA-DR T cells in patients with SLE (Kammer, 1999).

4.3.1.2: CD69.

This study is the first to investigate CD69 expression by DN T cells in patients with SLE. The differences between the percentages of $CD69^+$ DN T cell populations between SLE patients and controls, were much more significant than the differences seen comparing the $CD4/CD8^+$ populations, suggesting that the level of expression of CD69, although increased on the overall T cell populations was highest for the DN T cells in patients with SLE.

Other investigators have however, shown that numbers of $CD69^+$ cells are marginally increased in freshly isolated $CD4^+$ or $CD8^+$ T cells from patients with SLE, especially in the $CD8^+$ T cell compartment (Crispin *et al.*, 1998) (Fernandez-Gutierrez *et al.*, 1998; Sakata *et al.*, 1998; Portales-Perez *et al.*, 1997). In patients with RA, the percentage of $CD69^+$ and $HLA\text{-}DR^+$ T cells was found to be increased in the synovial fluid but not peripheral blood illustrating the restriction of activated T cells to the site of inflammation (Afeltra *et al.*, 1993; Bodman-Smith *et al.*, 2000). It has also been reported that CD69/CD3 ratio, which detects T cell activation, is correlated with disease activity in patients with SLE (Su *et al.*, 1997).

4.3.1.3: CD28.

More DN T cells expressed CD28 in patients with SLE compared to RA or HC. Ananlysis of the $CD4^+$ and $CD8^+$ cells together showed that there were significantly more $CD28^+$ cells in the DN compartment than in the $CD4/8^+$ population in patients with SLE. CD28 has been shown to be present on the majority of $CD4^+$ T cells with fewer $CD8^+$ T cells expressing this molecule (June *et al.*, 1994). Phenotype analysis studies of peripheral blood lymphocytes from patients with SLE revealed a decrease in the absolute number of $CD28^+$ T cells in both the $CD4^+$ and $CD8^+$ T cell compartments, while that of $CD28^-$ T cells was maintained (Garcia-Cozar *et al.*, 1996; Horwitz *et al.*, 1997; Kaneko *et al.*, 1996). The $CD28^+$ T cells from patients and healthy controls maintained a normal proliferative response to both CD28 dependent and independent stimulation, but the $CD28^-$ T cells were found to be hypo-responsive to anti-CD3 stimulation (Kaneko *et al.*, 1996). Decreased T cell response to anti-CD2 and its reversal by anti-CD28 antibody, observed in patients with SLE may be due to decreased CD28 mediated costimulatory activity following interactions of T cells with

conventional accessory cells (Horwitz *et al.*, 1997; Garcia-Cozar *et al.*, 1996). These results might explain in part the functional T cell defects observed in SLE. Conversely, other reports in the literature attribute T cell activation abnormalities in patients with SLE to irregularities in B7 expression on antigen presenting cells (Garcia-Cozar *et al.*, 1996; Denfeld *et al.*, 1997).

The significance of increased numbers of $CD28^+$ DN T cells in patients with SLE is currently unclear, but engagement of CD28 on T cells by its counter-receptors B7-1 (CD80) and B7-2 (CD86) on accessory cells is pivotal for T cell activation and in the absence of this costimulation T cells are anergised (Schwartz, 2003; Wells *et al.*, 2001). Recent studies in $CD28^{-/-}$ mice have also indicated an essential role for CD28 in Th2 differentiation and the initiation of T cell expansion in response to immunogenic antigen (Rulifson *et al.*, 1997; Howland *et al.*, 2000). Interestingly, a defective CD28/B7 costimulatory pathway has also been described in SLE T cells which could contribute to the disease pathogenesis (Horwitz *et al.*, 1997; Garcia-Cozar *et al.*, 1996).

4.3.1.4: CTLA-4.

Following activation via CD28, $CD4^+$ and $CD8^+$ T cells express CTLA-4, which has a higher affinity for the same ligands as CD28 (CD80/CD86). This expression is thought to deliver a negative signal to the T cells (Allison and Krummel, 1995; Waterhouse *et al.*, 1995; Tivol *et al.*, 1995). In these studies on CTLA-4 expression by CD4/$CD8^+$ T cells in patients with SLE increased percentages of CTLA-4^+ T cells were found compared to normal controls, consistent with that reported by others (Liu *et al.*, 1998). In addition, our patients with SLE also showed a low but significantly increased percentage of DN T cells expressing CTLA-4. This concomitant increase in the number of cells expressing both CD28 and CTLA-4 confirms the highly activated status of some DN T cells in patients with SLE.

CTLA-4 is often detected intracellularly. However, in this study I did not stain for intracellular CTLA-4 routinely because, in three patients studied I found no significant differences in the results for surface or intracellular staining (data not shown).(Garcia-Cozar *et al.*, 1996; Kaneko *et al.*, 1996)

4.3.1.5: CD45RA and CD45R0.

Expression of CD45R0 usually means that the cells are 'antigen experienced' whereas those that are CD45RA are 'antigen naïve'. I found that a significantly higher percentage of DN T cells expressed CD45RA in patients with SLE compared with HC. The opposite was found for the CD45R0 status, with significantly decreased percentages of $CD45R0^+$ DN T cells seen in patients with SLE compared to HC. The patients also showed a significantly higher proportion of $CD45RA^+$ cells to $CD45RO^+$ cells in the DN T cell compartment compared to the $CD4^+/CD8^+$ T cell population. One explanation for this could be the recruitment of naïve/resting DN T cells to the ongoing immune reaction in patients with SLE rather than the reactivation of existing memory clones. This finding is in agreement with the results of studies following the course of disease from the onset of symptoms in patients with SLE (Gordon *et al.*, 1996).

There was no significant increase in the percentages of $CD45RA^+$ DN T cells in patients with RA compared to HC. This is a surprising observation considering both patients with SLE and RA have ongoing inflammatory autoimmune reactions. This finding implies that the recruitment of DN T cells to autoimmune reactions differs in patients with varying autoimmune diseases. On the other hand, as RA is a relatively organ specific disease and newly recruited $CD45RA^+$ DN T cells could be present at the sites of inflammation (joints) and therefore the increased numbers not detected in the peripheral blood of patients.

4.3.2: DN T cells from patients with SLE are hypo-responsive to PHA stimulation.

In stimulation assays with a strong stimulant such as PHA, DN T cells from patients with SLE showed a lower response compared to cells from HC. Previous work monitoring activation of SLE lymphocytes in culture, after different stimuli has demonstrated that they show suboptimal proliferation and activation responses ((Alcocer-Varela *et al.*, 1991; Crispin *et al.*, 1998) (Portales-Perez *et al.*, 1997) (Horwitz *et al.*, 1997; Hernandez-Fuentes *et al.*, 1999). These studies on DN T cells agreed with these results, with the percentage increase in HLA-DR expression over 24 hours being significantly lower in DN T cells from patients with SLE compared to HC. This hypo responsiveness might be due to their already being highly activated when isolated from the peripheral blood of patients with SLE. Previous studies have demonstrated that the decreased proliferation response of SLE T cells can be restored by the addition of anti-CD28 MoAb suggesting the absence of an intrinsic defect in the activation and

proliferation pathways of these cells (Garcia-Cozar *et al.*, 1996). Interestingly, cell cycle analysis of activated (HLA-DR$^+$) T cells from patients with SLE by Takano et al., showed the majority to be present in the G1A phase with very few cells in the S, G2 or M phase (Tokano *et al.*, 1997). The authors concluded that there were at least two types of activated T cells in patients with SLE which differed in their cell cycle distribution (activated T cells in G1A phase or those in G1-M phase) and suggest that this variation may be related, perhaps indirectly to SLE pathogenesis.

4.4: Concluding remarks.

The data in this chapter suggests an activated phenotype for DN T cells from patients with SLE, indicating their participation in the ongoing immune reaction. The response of DN T cells from these patients to *in vitro* activation was lower than cells from HC, perhaps due to their already highly activated state in the peripheral blood of patients. The hyporesponsivenes of the DN T cells measured in these studies was consistent with similar observation by other groups looking generally at T cells.

Chapter 5.

Cytokine secretion by SLE DN T cells.

5.1: Introduction and aims.

5.1.1: Introduction.

5.1.1.1: Double negative T cells in SLE: Cytokine profile.

My studies on DN T cells have shown the presence of expanded populations of αβ TCR^+ DN T cell, and activated phenotypes in patients with SLE. The functional significance of these cells in the autoimmune reaction can be further understood by studying their cytokine production. The cytokine profile of these cells could indicate their role as Th1 (inflammatory), Th2 (humoral immune response), Th3 (regulatory function) or Th0 as described in detail in the general introduction.

Studies in the literature have shown that αβ TCR^+ DN T cell lines and clones from healthy subjects to have a higher proportion of IL-4 producing cells indicating that they may play a role in host 'Th2 like' responses, and provide help for antibody production (Katsikis *et al.*, 1995; Niehues *et al.*, 1999). αβ TCR^+ DN T cells from healthy subjects have also been shown to produce IFN-γ and IL-10 indicating a Th0 phenotype (Katsikis *et al.*, 1995). The production of both IL-4 and IL-10 by these cells could indicate an immunosuppressive and anti-inflammatory role for these cells in the autoimmune disease pathogenesis. In mice, a novel cell population called $NK1.1^+$ cells within the $CD4^+$ and DN T cell compartments associated with initial IL-4 production and Th2 differentiation has been reported, although equivalent cells in humans have not been clearly defined (Yoshimoto *et al.*, 1995). Recent studies on PBMC's from patients with SLE have reported higher frequencies of $IL\text{-}4^+$ T cells in both the $CD4^+$ and DN T cell compartment of active SLE patients compared to corresponding cells in healthy control subjects (Funauchi *et al.*, 1999).

5.1.1.2: Intracellular cytokines.

To determine cytokine production on the single-cell level, intracellular cytokine (IL-4) was determined by FACS. Cytokine synthesis was maximised by stimulating the cells with PHA and monensin which is a secretion inhibitor and allows the accumulation of cytokines in the Golgi bodies (Pala *et al.*, 2000). In general, measuring cytokines from plasma and serum

samples or culture supernatants (using techniques such as ELISA) does not reflect real *in vivo* conditions. Cytokines do not effectively regulate immune function systematically, but rather in low concentrations near the effector site. In addition, *in vitro* analyses of cytokine levels in supernatants from cultured cells are tempered by consuming cells within bulk cultures. All these factors can affect levels measured in the supernatant and hence the intracellular cytokine staining method was used in our studies (Jacob *et al.*, 2001).

5.1.2: Aims.

At the start of this project, nothing was known about the constitutive production of cytokines by DN T cells in patients with SLE. The aim of this study was to determine the percentages of IL-4 producing $\alpha\beta^+$DN T cells *ex vivo*, and following activation with PHA, from patients with SLE and controls and to compare these levels of cytokine production with those from the $CD4^+$ and $CD8^+$ $\alpha\beta$ TCR^+ T cell subpopulations.

5.2: Results.

5.2.1: Patients used in this study.

Fifty patients with SLE (49 females and 1 male aged 17 to 66 years, mean 37.2 years) were studied with informed consent. Each met four or more of the revised criteria of the American College of Rheumatology for the classification of the disease (Tan *et al.*, 1982). Disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) computerised index (Hay *et al.*, 1993). In this study patients with a global scoring >6 were deemed active ≤6 as inactive. Levels of circulating anti ds-DNA antibodies and C3 were measured during routine patient assessment. Serum levels of anti dsDNA antibodies in excess of 50 I.U. ml^{-1} (Shield Diagnostics, Dundee) and levels of C3 less than 0.9 I.U. ml^{-1} (by laser nephelometry) are regarded as abnormal. For statistical analysis in this study, anti-dsDNA antibody levels of ≥100 I.U. ml^{-1} or more were considered to be high. Detailed examination of the treatment regimes at the time of sampling showed that while many of the patients were on steroid therapy, either alone or in combination with other drugs, ten were receiving no major drug therapy.

Fifteen patients with rheumatoid arthritis (RA: 7 female, 8 male, aged 26 to 77 years, mean 50.7 years) who fulfilled four or more of the ARA criteria for rheumatoid arthritis and 16 healthy controls (HC: 13 females, 3 males aged 21 to 57 years, mean 36.1 years) were also studied (Arnett *et al.*, 1988).

Patient No	Global Score	Drug Treatment
1	2	5 mg Predisolone
2	5	4 mg Predisolone
3	2	5 mg Predisolone
4	4	5 mg Predisolone
5	3	5 mg Predisolone
6	4	2 mg Prednisolone
7	7	3 mg Predisolone
8	1	5 mg Predisolone
9	4	15 mg Predisolone
		Cell Sept 1500
10	4	10 mg Prednisolone
		150 mg Azathioprine
11	4	5 mg Predisolone
		15 mg weekly Methotrexate
12	3	3 mg mg Predisolone
13	1	5 mg mg Predisolone
14	15	7.5 mg mg Predisolone
15	4	5 mg mg Predisolone
16	7	10 mg Predisolone
		400 mg Hydroxychloroquine
17	1	7.5 mg Predisolone
		400 mg Hydroxychloroquine
18	7	10 mg Predisolone
		400 mg Hydroxychloroquine
19	5	10 mg Predisolone
		400 mg Hydroxychloroquine
20	9	8.5 mg Predisolone
		400 mg Hydroxychloroquine
21	2	2.5 mg Predisolone
		400 mg Hydroxychloroquine
22	2	400 mg Hydroxychloroquine
23	4	400 mg Hydroxychloroquine
24	2	400 mg Hydroxychloroquine
25	2	400 mg Hydroxychloroquine
26	5	400 mg Hydroxychloroquine
27	9	400 mg Hydroxychloroquine
28	2	400 mg Hydroxychloroquine
29	4	400 mg Hydroxychloroquine
30	1	200 mg Hydroxychloroquine
31	9	200 mg Hydroxychloroquine
32	2	400 mg Hydroxychloroquine
33	4	400 mg Hydroxychloroquine
34	10	5 mg Predisolone
		100 mg Azathioprine
35	8	5 mg Predisolone
		100 mg Azathioprine
36	1	5 mg Predisolone
		100 mg Azathioprine
37	3	5 mg Predisolone
		100 mg Azathioprine

38	7	5 mg Predisolone 100 mg Azathioprine
39	2	5 mg Predisolone 75 mg Azathioprine
40	2	5 mg Predisolone 100 mg Azathioprine
41	2	6 mg Predisolone 150 mg Azathioprine
42	2	6 mg Predisolone 150 mg Azathioprine
43	6	5 mg Predisolone 125 mg Azathioprine
44	6	7.5 mg Predisolone 100 mg Azathioprine
45	5	15 mg Predisolone 150 mg Azathioprine 400 mg Hydroxychloroquine
46	5	9 mg Predisolone 400 mg Hydroxychloroquine
47	4	50 mg Azathioprine 200 mg Hydroxychloroquine
48	5	10 mg Azathioprine 400 mg Hydroxychloroquine 5 mg weekly Methotrexate
49	7	100 mg Azathioprine
50	2	12.5 mg Predisolone 400 mg Hydroxychloroquine 10 mg weekly Methotrexate

Table 5.1: Disease activity (Global scores) and treatments of patients with SLE studied in this chapter.

5.2.2: Production of IL-4 by αβ$^+$DN, CD4$^+$ and CD8$^+$ T cells.

a) Constitutive levels of IL-4.

αβ TCR$^+$DN T cells isolated from patients with SLE, constitutively had significantly higher percentages of IL-4 containing cells than patients with RA ($p = 0.003$, Fig.5.1). The difference between patients with SLE and HC was not significant ($p = 0.066$). However, 12 of the patients with SLE were shown to have a percentage of αβ TCR$^+$DN T cells containing IL-4 of more than twice the standard error of the mean of HC (Fig. 5.1). The number of patients with SLE who showed high percentages of IL-4 containing cells was significantly different from HC by Fischer's exact test ($p = 0.025$). In addition, it was observed that the percentages of IL-4 positive cells were significantly higher in the αβ TCR$^+$ DN T cell subset than in either the CD4$^+$ or the CD8$^+$ T cell subsets of patients with SLE both in whole population analyses ($p = <0.0001$ in each case) and in the 12 patients with IL-4 positive cells greater than twice the standard error of the mean of HC ($p = 0.006$ for CD8$^+$ and $p = 0.019$ for CD4$^+$). It was difficult to determine whether increased numbers of IL-4$^+$ αβ TCR$^+$ DN T cells in patients compared with controls also showed these individual cells to be producing more IL-4 (by measuring the mean fluorescence intensity: MFI of IL-4 staining) owing to variations in binding of isotype controls and anti-IL-4 antibody between patients.

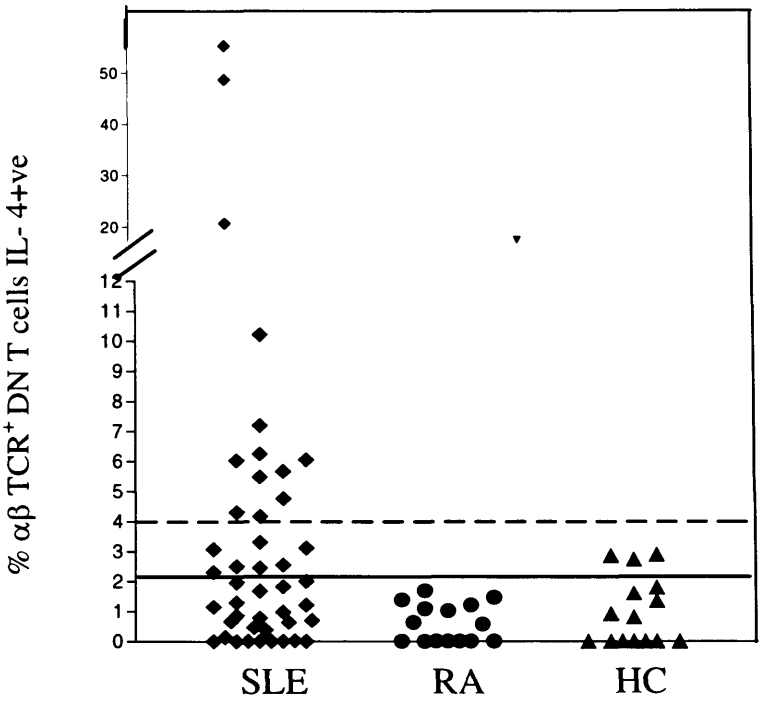


Figure 5.1: IL-4 production by αβ TCR^+ DN T cells in patients with SLE (n=50), RA (n=15) and HC (n=15). The mean is indicated by solid line and the dotted line represents +2 x SEM of patients with SLE. Enriched DN T cells were identified by immunofluorescence staining with antibodies to αβ TCR (FITC) (FL1) and CD4 and CD8 (CyChrome) (FL3). Cells were further stained with fluorochrome (PE) conjugated antibodies to intracellular IL-4 and analyzed by flow cytometry as described in materials and methods. An electronic gate was placed around αβ TCR^+ DN T cells and their intracellular IL-4 expression analyzed.

b) IL-4 production after PHA stimulation.

When cells from these experimental groups were stimulated by overnight incubation with PHA, the differences in frequency of IL-4 positive cells between the groups were no longer observable (Fig 5.2). Analysis of paired data from constitutive and PHA stimulated cells showed a significant increase in the percentage of IL-4 positive αβ TCR^+DN T cells on stimulation for SLE (p=0.03), RA (p=0.006) and HC (p=0.004). There was no significant difference in the percentage IL-4 positive cells either between SLE and RA (p=0.8) or between SLE and HC (p=0.9). It was noted that, while most individuals tested showed an

increase in IL-4 positive cells following PHA stimulation, six of the patients with SLE decreased their percentages of IL-4 positive αβ TCR^+ DN T cells after stimulation.

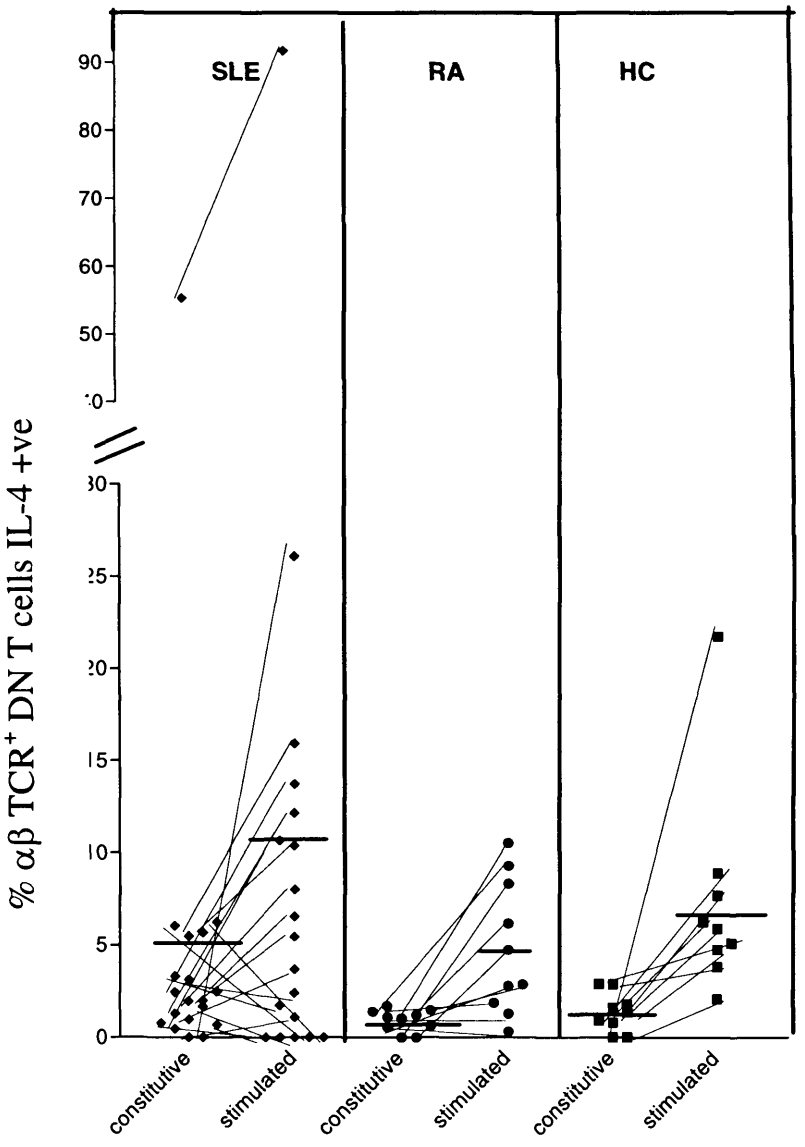


Figure 5.2: αβ TCR^+ DN T cells from patients with SLE (n=19), RA (n=10) and healthy controls (n=9) expressing IL-4 before and after stimulation with PHA *in vitro*. The mean in each set of data points is indicated by the horizontal solid line. Enriched DN T cells were identified by immunofluoresence staining with antibodies to αβ TCR (FITC) (FL1) and CD4 and CD8 (CyChrome) (FL3). Cells were further stained with flurochrome (PE) conjugated antibodies to intracellular IL-4 before and after stimulation with PHA, and

analyzed by flow cytometry as described in the materials and methods. An electronic gate was placed around αβ TCR^+ DN T cells and their intracellular IL-4 expression analyzed.

5.2.3: Quantitation of αβ TCR^+ double-negative T cells.

Previous studies (described in chapter 3) indicated that the αβ TCR^+DN T cell subpopulation was expanded within the total DN population in patients with SLE. While the total lymphocyte population comprised of approximately 1%-12% DN T cells in all experimental groups, an average of 60% of these cells were αβ TCR^+ in patients with SLE compared with an average of 48% in healthy people (p=0.0216) (Fig 3.6). Comparison of percentages of αβ TCR^+DN T cells as a proportion of total αβ TCR^+ T cells for those 15 patients who had the highest frequency of IL-4 positive αβ TCR^+DN T cells with percentage of $\alpha\beta^+$DN T cells for those patients with low frequency of IL-4 positive cells, showed that those patients with high percentages of IL-4 positive cells had significantly fewer αβ TCR^+ T cells in the DN compartment than did the group with low frequency of IL-4 positive cells (p=0.02) (Figure. 5.3). However, further analysis showed that there was no overall correlation between percentages of IL-4 positive $\alpha\beta^+$DN T cells and total $\alpha\beta^+$DN T cells (r=-0.15, p=0.36).

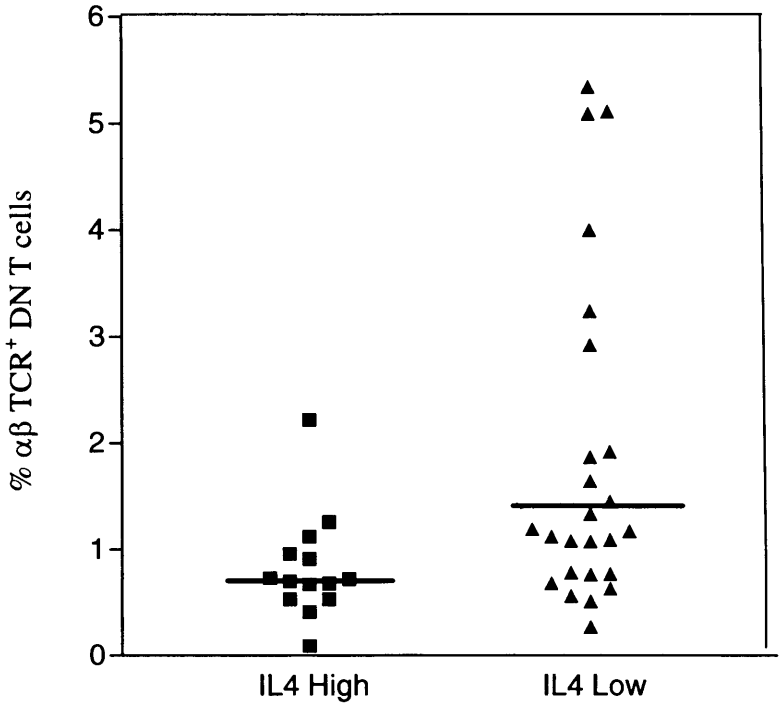


Figure 5.3: Percentages of αβ TCR^+ DN T cells in patients with high (n=14) and low (n=25) numbers of IL-4 producing cells. The mean is indicated by the horizontal solid line. Enriched DN T cells were identified by immunofluoresence staining with antibodies to αβ TCR (FITC) (FL1) and CD4 and CD8 (CyChrome) (FL3). Cells were further stained with fluorochrome (PE)(FL 2) conjugated antibodies to intracellular IL-4 and analyzed by flow cytometry as described in materials and methods. An electronic gate was placed around αβ TCR^+ DN T cells and their intracellular IL-4 expression analyzed.

5.2.4: No correlation of IL-4 production with cell surface markers

Levels of the surface activation markers CD69 and HLA-DR were measured on αβ TCR^+DN T cells from 21 patients with SLE, 17 of whom had low frequencies of IL-4 positive cells and four had high frequencies of IL-4 positive αβ TCR^+DN T cells. There was no

significant difference in activation marker expression between high and low producers of IL-4 (p= 0.51 for HLA-DR and p=0.58 for CD69) (Fig.5.4). In addition, there was no significant correlation between expression of activation markers and high or low frequency of IL-4 positive $\alpha\beta^{+}$DN T cells (r=-0.12, p=0.33 for HLA-DR and r=0.78, p=0.39 for CD69) (Fig5.4). In the above experiments, DN T cells were independently evaluated for activation markers and IL-4 expression. It was not possible to determine whether the IL-4 containing cells themselves expressed HLA-DR or CD69 in this study owing to the technical limitations of three colour staining.

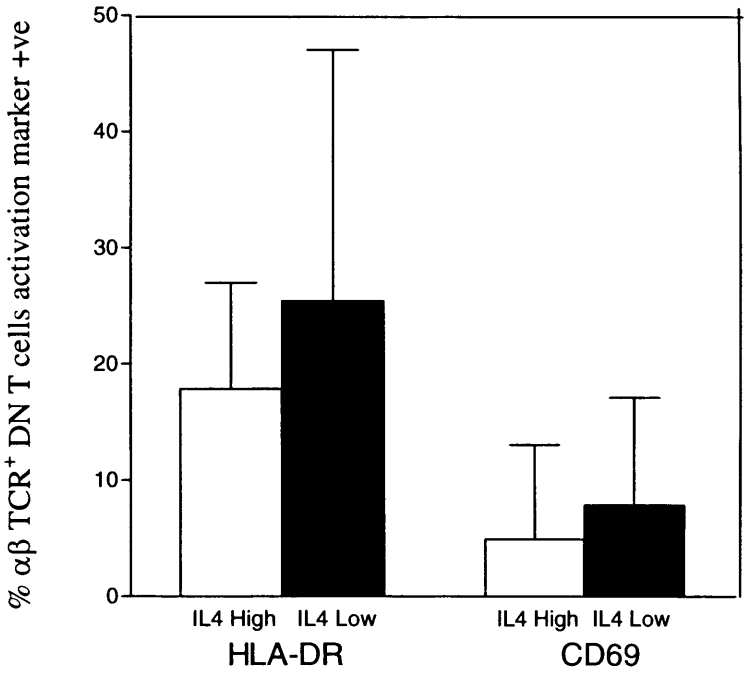


Figure 5.4: Relationship between activation marker expression and numbers of high (n=4) and low IL-4 (n=17) expressing αβ TCR^{+} DN T cells in patients with SLE. Enriched DN T cells were identified by immunofluorescence staining with antibodies to αβ TCR (FITC) (FL1) and CD4 and CD8 (CyChrome) (FL3). Cells were further stained with flurochrome (PE) conjugated antibodies to intracellular IL-4 or surface HLA-DR or CD69 and analyzed by flow cytometry as described in materials and methods. An electronic gate was placed around αβ TCR^{+} DN T cells and their intracellular IL-4 or HLA-DR or CD69 expression analyzed.

5.2.5: IL-4 production and disease activity.

There was no correlation between the BILAG Global Disease Activity Index and IL-4 production (r=0.07, p=0.6). Similarly, when the patients with SLE were divided into groups depending on the specific organs or systems affected, there was no connection between IL-4 production and the organ or system involved. It was not possible to calculate correlation coefficients for each of the specific organs or systems owing to the small sample size.
The 12 patients in the high constitutive IL-4 group had a mean disease duration of 10.67 years ± 2.64 years. The low IL-4 group consisted of 29 patients of mean disease duration 12.64 years ± 1.56 years. There was no significant difference in disease duration between the two groups (p= 0.6).

5.2.6: No association of constitutive IL-4 production with drug treatment.

All but one of the patients with SLE who showed high levels of IL-4 positive αβ TCR^+DN T cells was receiving prednisolone therapy (dosage varying from 3-10mg). Five of these patients were also being treated with azathioprine (dosage varying from 50 -100mg). The remaining patient was being treated with antimalarial drugs (Hydroxychloroquine, 400mg). Twenty four of the low IL-4 group were also being treated with prednisolone, eleven of these in combination with azathioprine and one in combination with antimalarials. One patient was receiving azathioprine alone, seven antimalarials alone, and one patient was being treated with a combination of antimalarials and azathioprine. There was no association between drug treatment and levels of constitutive production of IL-4. When the patients treated with prednisolone were divided arbitrarily on the basis of steroid dosage into high and low dose groups (≥ 10mg and <10mg respectively) no significant differences in percentages of IL-4 positive αβ TCR^+DN T cells or percentages of αβ TCR^+DN T between the two groups were found (p= 0.77 and p=0.84 respectively).

5.2.7: IL-4 production did not correlate with levels of plasma C3 or circulating anti dsDNA antibodies in patients with SLE.

Circulating anti dsDNA antibodies are a characteristic of patients with SLE and rising levels of antibody are often a predictor of severe disease. In the 47 patients for whom data were available, there was no correlation between levels of circulating anti dsDNA antibodies and the percentages of IL-4 positive $\alpha\beta^{+}$DN T cells (r=0.07 p=0.66).

Plasma C3 is measured routinely in all patients attending the Lupus clinic. Low levels of C3 often correlate with active disease. However, in 47 patients for whom data were available, percentages of $\alpha\beta^{+}$DN T cells constitutively expressing IL-4 did not correlate with plasma C3 (r=0.21, p=0.44)

However, there was a statistically significant negative correlation between C3 and anti dsDNA for the patient samples considered in this study (r=-0.45 p=0.01) confirming the known relationship between high anti dsDNA antibodies and low C3.

5.2.8: No association of IL-4 production with HLA phenotype.

Tissue-typing data were not available for all of the patients but were obtained for nine of the high frequency IL-4 positive group and for 24 of the low frequency group. There was a wide variation in HLA expression but it was noted that several phenotypes occurred multiple times. Five of the 24 patients in the low frequency group expressed HLA A1 and HLA B8 while another five expressed HLA DQ5. In the high frequency group, four of the nine patients expressed each of these phenotypes. Fischer's exact test indicated that the differences in expression between the two groups were not significantly different (p= >0.5) as might be expected, given the relatively small sample number.

5.2.9: Summary of results from chapter 5.

The data from this chapter can be summarized as follows:

i. The number of constitutively IL-4 expressing αβ TCR^+ DN T cells was significantly greater in patients with SLE than patients with RA(p=0.003). The difference between patients with SLE and HC was not significant (p=0.066).

ii. The percentage of IL-4 positive cells was significantly higher in the αβ TCR^+ DN T cell subset than in either the $CD4^+$ or the $CD8^+$ T cell subsets of patients with SLE (p=<0.0001 in each case).

iii. Twelve patients with SLE showed a percentage of IL-4^+ αβ TCR^+ DN T cells of more than twice the standard error of mean of this population of cells in HC.

iv. On stimulation of αβ TCR^+ DN T cells with PHA, no difference in the numbers of IL-4 positive cells was found either between patients with SLE and RA (p=0.8) or between those with SLE and HC (p=0.9). All the groups (SLE, RA and HC) showed increased numbers of IL-4 positive cells after PHA stimulation compared to freshly isolated αβ TCR^+ DN T cells (SLE p=0.03, RA p=0.006 and HC p=0.004).

v. Patients with SLE who had high percentages of IL-4 positive cells had significantly fewer αβ TCR^+ T cells in the DN compartment than did the group with a low frequency of IL-4 positive cells (p=0.02).

vi. There was no relationship between expression of activation markers (CD69 and HLA-DR) and high and low IL-4 expression by αβ TCR^+ DN T cells in patients with SLE (p= 0.51 for HLA-DR and p=0.58 for CD69).

vii. Levels of constitutive IL-4 expression by αβ TCR^+ DN T cells of patients with SLE showed no association with disease activity, drug treatment, levels of plasma C3 or circulating anti-dsDNA antibodies.

5.3: Discussion

In this chapter I have shown that the αβ TCR^+DN T cell compartment of patients with SLE contains more cells positive for intracellular IL-4 than the equivalent cells of RA autoimmune controls or healthy controls. These results are consistent with the results of Sieling et al., who have also reported increased numbers of IL-4 producing αβ TCR^+ DN T cells in patients with SLE (Sieling *et al.*, 2000). Other groups have also reported increased production of IL-4 by T cells from patients with autoimmune and atopic diseases(Nagy *et al.*, 2000 ; Funauchi *et al.*, 1998; Richaud-Patin *et al.*, 1995). Since these studies only looked at IL-4 and not IFN-γ production, I was unable to determine whether the IL-4 producers were also IFN-γ^+. Intracellular staining with a number of anti-IFN-γ antibodies failed to give consistent staining patterns on enriched DN T cell populations making the data difficult to interpret and was therefore abandoned.

Funauchi et al, have demonstrated that the frequency of IL-4 positive cells is increased in SLE compared with IFN-γ producers and it has been shown that IL-4 enhances the differentiation of both B cells and T cells, particularly Th2 helper T cells (Funauchi *et al.*, 1998). Recent studies in murine lupus, have advocated a protective role for IL-4 producing cells in down regulating the production of Th1 mediated IgG3 subclass of autoantibodies, which have been shown to be more nephritogenic (Santiago *et al.*, 1997).

A higher frequency of NK T cells (a subpopulation of αβ$^+$DN T cells) producing IL-4 in patients with SLE than controls, following stimulation with PMA and ionomycin has been described (Funauchi *et al.*, 1999). This NK T population mainly utilizes Vα24JαQVβ11+ T cell receptor in healthy subjects and these cells have been shown to produce both IL-4 and IFN-γ on activation. In insulin dependent diabetes mellitus (IDDM) it has been reported that Th1 cell-mediated tissue damage is initially regulated by Vα24JαQVβ11^+ T cells producing both IFN-γ and IL-4 cytokines, and loss of their capacity to produce IL-4 is correlated with IDDM (Wilson *et al.*, 1998). This population of Vα24JαQVβ11^+ DN T cells has been shown

by others to be reduced in patients with active SLE is dysfunctional in about 50% of patients although DN Vα24$^+$ T cells are expanded overall (Oishi *et al.*, 2001 ; Kojo *et al.*, 2001). When these patients become inactive with the help of prednisolone therapy, patients on therapy regain their Vα24JαQVβ11$^+$ DN T cells but it is unclear as to whether their function is restored. This finding would implicate Vα24JαQVβ11$^+$ DN T cells in control of the pathogenesis of SLE possibly through the production of IL-4 (Kojo *et al.*, 2001). In general, it has been suggested that IL-4 producing NK T cells might function as regulatory cells, and the selective reduction of this population may be essential for the generation of autoimmune diseases.

It is unclear at present why only a minority of patients had an increased frequency of intracellular IL-4 in the αβ TCR$^+$DN subpopulation, suggesting that these patients are in some way different from others within the group. In addition, a high frequency of intracellular IL-4 appeared to be specific to the αβ TCR$^+$DN T cell population and was not found in the CD4$^+$ and CD8$^+$ T cell populations. Attempts to correlate the increased expression of IL-4 by the αβ TCR$^+$DN T cell population with a variety of other criteria failed to yield any relationships. These patients were not from a common ethnic background, did not share a common HLA phenotype and there was no relationship between the severity of their disease at the time of sampling and the percentage of IL-4 positive cells. This last observation is in contrast to the work of another group who found that disease activity was lowest in those patients who produced the highest levels of IL-4 (Funauchi *et al.*, 1999). Indeed, the highest levels of IL-4 in their patient cohort were produced by patients with a SLEDAI global score of one. However, their data was only generated from the subpopulation of DN cells expressing CD57 (NK T cells) and not from the whole αβ$^+$DN T cell population and so may not be comparable with our findings. There appeared to be no relationship between levels of plasma C3 and anti dsDNA antibodies and IL-4 produced suggesting that the high levels of intracellular IL-4 in some patients are not simply related to disease activity using these criteria. In addition, there appeared to be no obvious relationship between drug therapy and production of IL-4. Interestingly, almost all of our patients were being treated with prednisolone.

Further analysis of the data showed that the percentages of αβ TCR$^+$DN T cells present in the 15 patients with the highest frequency of IL-4 containing cells was significantly lower than that in the other patients with SLE. Although the significance of this observation is not

yet clear, it may lend weight to the theory that high levels of IL-4 within the DN T cell population are seen in those patients who lack a regulatory population of cells that would otherwise control cytokine production. Recent reports have also suggested a role for IL-4 producing cells in containing Th1 (IFN-γ) mediated production of nephritogenic subclass of IgG autoantibodies (Kirou and Crow, 1999; Theofilopoulos *et al.*, 2001). Alternatively, it is possible that when the αβTCR$^+$DN T cells are present in lower numbers, they receive more help to produce cytokines. Such a possibility would suggest that the IL-4 producing cells would be more activated and measurement of activation markers on the whole subpopulation of αβ TCR$^+$DN T cells appeared to confirm this theory though the difference was not significant and numbers were too low to draw firm conclusions. However, It was not possible to determine whether those cells positive for intracellular IL-4 were also activated within the constraints of three-colour staining and further work would be needed to clarify this point. Since IL-4 is under genetic control and polymorphisms have been shown to be related to its production, it would be valuable in future studies to determine any differences in the genotype in relation to IL-4 production (Kanemitsu *et al.*, 1999). The increased receptor function seen in some patients with SLE may play a role in the development of disease through induction of Th2 subset development.

The observation that stimulation with PHA masked the differences observed between the experimental groups may be explained by the fact that cells in patients with SLE are already in an activated state and have been shown not to react strongly to mitogenic stimulation (Hernandez-Fuentes *et al.*, 1999). Stimulation with PHA caused most of the individuals tested to have a higher percentage of IL-4 positive αβ TCR$^+$ DN T cells although there appeared to be a lower level of stimulation in the patients with SLE. Indeed, six of these patients could be seen to have a lower percentage of IL-4 positive DN T cells than they expressed constitutively. It is unclear as to why these six patients had reduced levels of IL-4 containing cells since there was no apparent correlation following PHA stimulation with disease activity, disease duration, or percentage of αβ TCR$^+$ DN T cells. In addition, these six patients were not concordant for drug treatment and did not appear to be concordant for HLA phenotype.

5.4: Concluding remarks

In conclusion, the data in this chapter indicates that in about one third of patients with SLE, there is a higher frequency of αβ TCR^+DN T cells that constitutively express IL-4 than in control individuals. This increase in IL-4 was more accentuated in the αβ TCR^+DN T cells then in the $CD4^+$ and $CD8^+$ T cell populations, suggesting that the DN T cell compartment could be functionally more important than the conventional $\alpha\beta^+$ T cells in these patients with SLE. Recent studies in mice and patients with SLE have indicated a regulatory /immunosupressive role for IL-4^+ Vα24JαQVβ11^+ DN T cells and conventional IL-4 producing T cells in containing Th1 mediated pathogenic autoantibody response.

Chapter 6.

Apoptosis in SLE DN T cells.

6.1: Introduction and aims.

The process of programmed cell death (PCD) or apoptosis is thought to play a central role in the pathogenesis of SLE by providing an immunogenic stimulus for autoimmunity. Defects in the apoptotic process can also lead to impaired activation induced cell death (AICD) and impaired tolerance to self-antigens, also contributing to the pathogenesis of autoimmune diseases such as SLE.

Double negative T cells have been implicated, as contributing to the disease pathogenesis of SLE, in a number of studies. The main roles proposed for their involvement is as helper T cells or regulatory/suppressor cells. Many investigators have studied apoptosis and apoptosis related molecules in conventional ($CD4^+$ or $CD8^+$) T and B cells of SLE patients. The DN T cell population has largely been ignored in these studies. In view of their potential contribution to the disease pathogenesis in SLE, the process of apoptosis in these cells merits study.

6.1.1: Introduction.

6.1.1.1: Apoptosis in SLE.

The immunological pathogenesis of SLE is multifactorial but the precise details remain elusive. Several clues from animal models of autoimmunity suggest that dysregulation of apoptosis can lead to clinically overt autoimmune phenomena. Until recently, the importance of these mechanisms in the development of human autoimmune diseases remained uncertain. Recent advances in the field of apoptosis have suggested new paradigms for the development of lupus autoimmunity.

Apoptosis plays an important role in the homeostasis of the immune response (Cohen, 1993). It can be induced by the interaction of the Fas receptor, a member of the TNF receptor family and regulated by the Bcl-2 family of genes. Apoptosis is defective in MRL lpr/lpr mice (which do not express a functional Fas receptor) and gld/gld mice (which have defective expression of the Fas ligand). Both murine strains develop autoimmune syndromes that have

similarities to human SLE (Cohen and Eisenberg, 1992). However, characteristic mutations similar to those reported in the MRL lpr/lpr or gld/gld mice have not been identified in patients with SLE. On the other hand, defects in the apoptotic pathway involving Fas-Fas-L interactions and the *bcl-2* family of genes, have been identified in some patients with SLE.

Early reports indicating increased numbers of circulating apoptotic cells in the peripheral blood of SLE patients, have not been substantiated by later studies as being specific to SLE (Emlen *et al.*, 1994; Perniok *et al.*, 1998) (Lorenz *et al.*, 1997; Courtney *et al.*, 1999). Reports indicating impaired phagocytosis of apoptotic cells might provide a clue to the persistence of apoptotic cells in circulation, in SLE patients (Herrmann *et al.*, 1998; Baumann *et al.*, 2002).

Links between Bcl-2 expression and autoimmunity were established by observations in mice with *bcl-2* transgenes over expressed in their B cells. These mice which developed an autoimmune syndrome, with many features of human SLE, had an excess of mature B-cells which showed extended survival (McDonnell *et al.*, 1989; Strasser *et al.*, 1991). Bcl-2 is an intracellular, apoptosis-inhibitory protein thought to block apoptosis due to its function in an antioxidant pathway and an oxygen independent pathway (Hockenbery *et al.*, 1990). Bcl-2 and the Fas/APO-1 system seem to be at least in part, functionally linked, since it has been shown that Bcl-2 signals are able to inhibit Fas induced apoptosis (Itoh *et al.*, 1993). The phenotype of Bcl-x_L transgenic mice is essentially indistinguishable from that of a Bcl-2 transgenic animal (Chao *et al.*, 1995).

As discussed in the general introduction, expression of the bcl-2 proto-oncogene has been extensively studied in the lymphocyte population of patients with SLE, with contradicting results. Increased expression of the Bcl-2 m-RNA and protein in SLE lymphocytes and sera has been observed in patients with SLE although, this would directly contradict reports of increased apoptosis (Liu *et al.*, 1998; Gatenby and Irvine, 1994) (Miret *et al.*, 1999; Falcini *et al.*, 1999) (Aringer *et al.*, 1994). Other investigators report unchanged or decreased levels of Bcl-2 expression in SLE lymphocytes *in-vivo* (Chan *et al.*, 1997) (Rose *et al.*, 1995; Lorenz *et al.*, 1997). There have been no conclusive reports on the other pro and anti- apoptotic members of the Bcl-2 family such as Bax, Bcl-x, bak etc.

6.1.2: Aims.

SLE has been associated with a dysregulation of the apoptotic machinery. In view of previous studies and these studies, which have shown a possible association of DN T cells with the pathogenesis of SLE, I decided to study the regulation of apoptosis in the DN T cell population of patients with SLE and healthy controls.

The questions I set out to answer were:

- Do constitutive levels of apoptosis in the DN T cell population of patients with SLE, differ from conventional T cells?
- Do constitutive levels of apoptosis differ betwwen the DN T cell populations of patients with SLE and healthy controls?
- Are the rates of apoptosis different between the DN T cell populations of patients with SLE and healthy controls, when cells are cultured *in-vitro*?
- Is the expression of Bcl-2 and Fas molecules changed between the DN T cell populations of patients with SLE and healthy controls?
- Is the rate of apoptosis in the DN T cells associated with the Bcl-2/Bax ratio, in patients with SLE patients and healthy controls?
- Is there an association between the expression levels of Bcl-x in the DN T cells of patients with SLE and healthy controls, and the rate of apoptosis?

6.2: Results.

6.2.1: Patients used in this study.

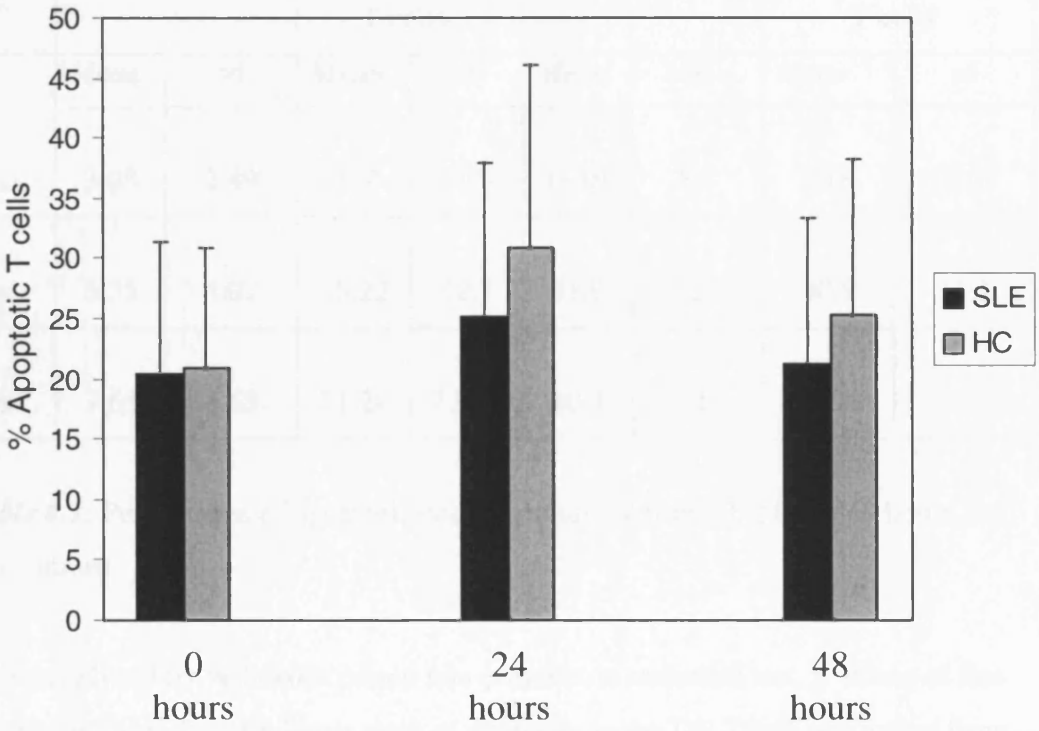
Fifteen patients with SLE were used in this study. All the patients were female. The mean age was 39 years and the range 20 to 65 years. Disease activity was assessed using BILAG index. A global score was determined as described in the Introduction and patients scoring >6 were deemed active and ≤ 6 inactive. Fifteen patients with rheumatoid arthritis (12 female, 3 male aged 27 to 75 years, mean 51 years) who fulfilled four or more of the ARA criteria for rheumatoid arthritis were also studied (Arnett *et al.*, 1988).

The 15 healthy controls studied were laboratory staff and female. The mean age was 31 years and the range 20 to 45 years.

6.2.2: Apoptosis in conventional ($CD4^+/CD8^+$) T cells of patients with SLE and healthy controls.

Apoptosis in cells was analyzed by annexin V- FITC staining as described in chapter 2 (Pepper *et al.*, 1998). Annexin V identifies cells at an early stage of apoptosis by binding to membrane phosphatidyl serine.

The $CD4^+/CD8^+$ T cell population of SLE patients and healthy individuals showed no differences in the levels of apoptosis in cells, freshly isolated from peripheral blood (p=0.91). The rate of apoptosis of the cells in culture, over 48 hours, did not differ significantly between SLE $CD4^+/CD8^+$ T cells and those of healthy controls (0-24 hrs p=0.3, 24-48 hrs p=0.47). The results are shown in figure 6.1.



Fiure 6.1: Percentage apoptotic ($CD4^+/CD8^+$) T cells in patients with SLE (solid bars) (n=10) and healthy controls (hatched bars) (n=7) at times 0, 24 and 48 hours in culture. The bars represent the mean ± standard deviation. PBMCs in *in-vitro* cultures were stained with CD3 (PE) and Annexin V (FITC) at times 0, 24 and 48 hours. An electronic gate was placed around T cells ($CD3^+$) and gated cells analyzed for annexin V binding.

6.2.3: Apoptosis in DN T cells of patients with SLE and healthy controls.

Enriched double negative T cells were isolated from patients with SLE and healthy controls as described in chapter 2. DN T cells were cultured for different periods of time (0, 24 and 48 hrs). At each time point cells were harvested and apoptotic cells quantitated.

Both the constitutive levels of apoptosis, and rate of apoptosis in culture of DN T cells in both SLE patients and healthy controls was significantly lower than in $CD4^+$/ $CD8^+$ T cells as shown in table 6.1.

	SLE n=10				HC n=7			
Time	DN T cells		$CD4^+/CD8^+$ T cells		DN T cells		$CD4^+/CD8^+$ T cells	
	Mean	sd	*Mean*	sd	*Mean*	sd	*Mean*	sd
0 hrs	3.05	2.49	20.4	10.9	11.01	8.2	20.9	10.07
24 hrs	6.35	4.07	25.22	12.7	21.9	12.8	30.9	15.1
48 hrs	7.65	4.65	21.28	12.07	10.7	5.12	25.28	12.9

Table 6.1: Percentages of apoptotic cells in culture at times 0, 24 and 48 hours (sd: standard deviation).

When analyzed by Wilcoxon paired non-parametric statistical test, p values of less than 0.05 indicated significantly lower rates of apoptosis in the DN T cell population from both patients with SLE and HC. The p values are shown in table 6.2.

	DN T cells < $CD4^+/CD8^+$ T cells p values	
	SLE	HC
Day 0	0.0039	0.015
Day 1	0.002	0.04
Day 2	0.002	0.0156

Table 6.2: P-values for the lower cell death in DN T cell population from both SLE patients and healthy controls.

In addition, freshly isolated SLE DN T cells showed lower levels of apoptosis compared to DN T cells from healthy controls, although, the difference did not reach statistical significance (p=0.0639).

After 24 hrs in cell culture, DN T cells from patients with SLE still showed significantly lower levels of apoptosis (6.35±4.07 % annexinV^+ cells) compared to cells from healthy controls (21.95± 12.8 % annexin V^+ cells) (p=0.0068).

Harvested after 48 hrs in culture, DN T cells from both SLE patients and HC exhibited similar levels of apoptosis (p=0.26). These results are shown in figure 6.2.

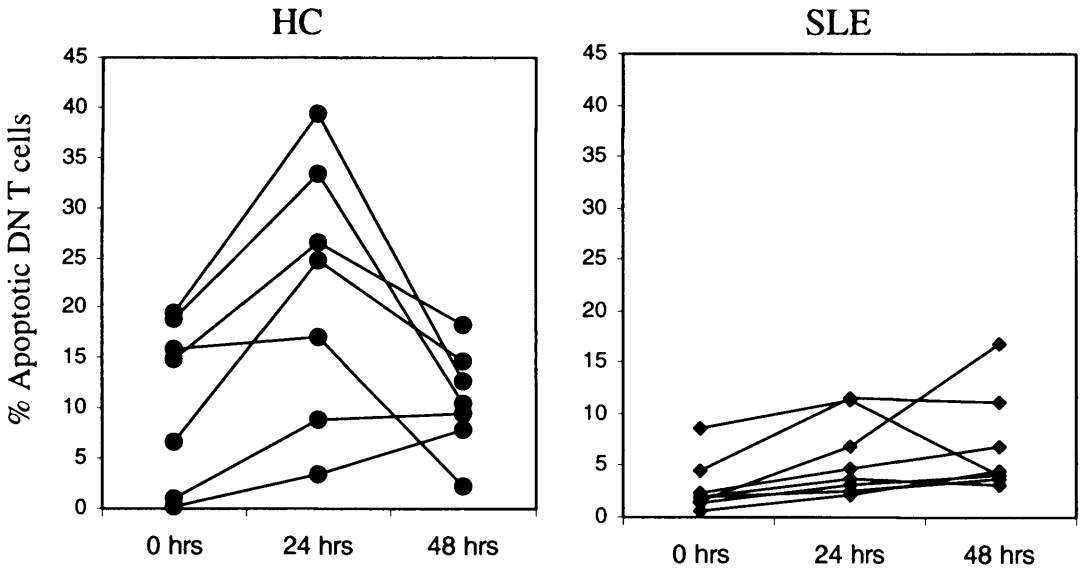


Figure 6.2: Programmed cell death in DN T cells on culture over 48 hours in patients with SLE (n=8) and healthy controls (HC: n=7). Enriched DN T cells were were cultured and

identified by flow cytometric staining with antibodies to CD3 (PE) (FL2), CD4 and CD8 (CyChrome) (FL3). Apoptosis at 0, 24 and 48 hours was measured by annexin V (FITC) (FL1) staining. Cells gated on a DN ($CD3^+$ $CD4^-$ $CD8^-$) T cell gate were analyzed for annexinV binding.

6.2.4: There was no difference in the expression of surface Fas molecules between patients with SLE, RA and HC.

To understand if differential expression of Fas could play a role in the decreased levels of apoptosis seen in the DN T cells of patients with SLE, we studied Fas expression on the surface of these cells. As shown in figure 6.3, DN T cells from SLE patients showed no increase in spontaneous Fas expression compared to cells from RA patients or healthy controls (p=0.9). The density of surface expression of Fas, as indicated by the mean fluorescence intensity of Fas staining, also showed no difference between patients with SLE and RA and HC DN T cells.

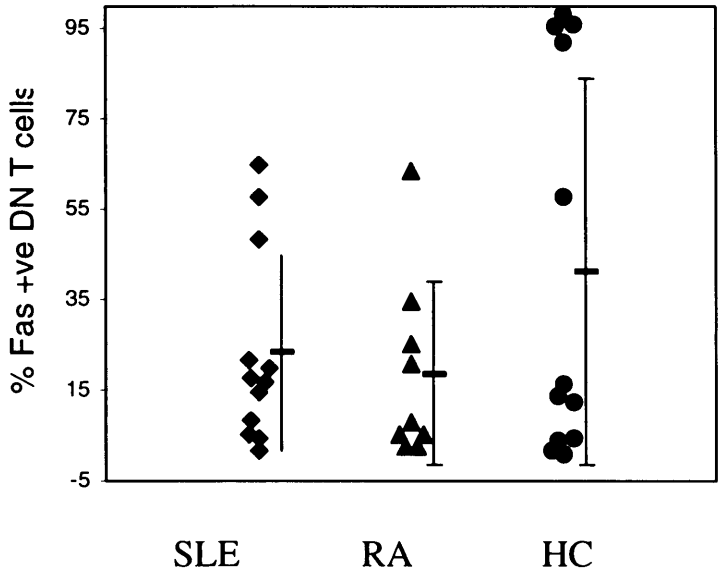


Figure 6.3: Fas^+ DN T cells in patients with SLE (n=12), RA (n=9) and healthy controls (n=12) (p= 0.9). Enriched double negative cells identified by staining with antibodies to CD3 (PE), CD4 and CD8 (CyChrome), were examined for surface Fas expression by staining with an anti-Fas (FITC) antibody.

6.2.5: Bcl-2 family of proteins: expression in double negative T cells.

I analyzed the expression of apoptosis related gene products at the protein level in DN T cells from patients with SLE and HC. The gene products studied were: Bcl-2, Bax, and Bcl-x. Enriched DN T cells from SLE patients and HC were cultured *in-vitro* in complete medium, over a 48-hour period. Cells were harvested at each time point (0, 24 and 48 hrs) and intracellular staining with Bcl-2, Bax or Bcl-x antibody was performed as described in Chapter 2. Representative histograms are shown in figure 6.4.

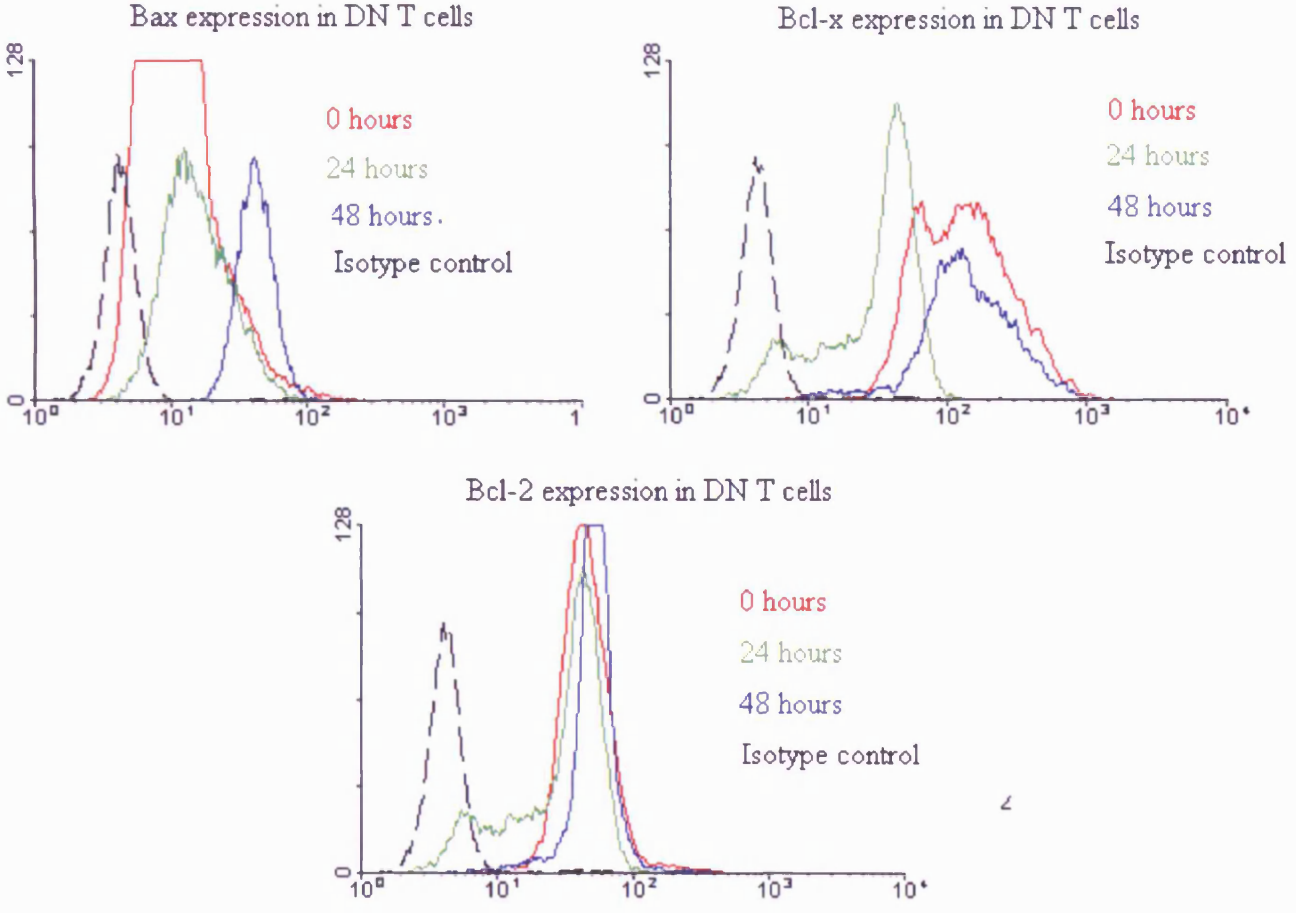


Figure 6.4: Representative histograms of DN T cells from a patient with SLE stained for Bax, Bcl-x and Bcl-2 proteins using intracellular staining techniques described in chapter 2 (materials and methods).

6.2.6: Bcl-2 expression.

The studies on the levels of constitutive apoptosis in DN T cells showed significantly decreased apoptosis in DN T cells of patients with SLE. Since the Bcl-2 protein is anti-apoptotic in nature, I examined the expression of this protein in both the total T lymphocyte and the DN T cell populations of patients with SLE, RA and HC.

The numbers of Bcl-2^+ T cells remained unchanged between patients with SLE, RA and HC ($p= 0.12$). Also, there were no significant differences in the numbers of Bcl-2^+ DN T cells between patients with SLE, RA and HC ($p=0.367$). These results are shown in figure 6.5. We also examined the mean fluorescence intensity (MFI) of Bcl-2 staining in DN T cells from patients with SLE, RA and HC and found no differences in the MFI values ($p= 0.24$), as seen in figure 6.6.

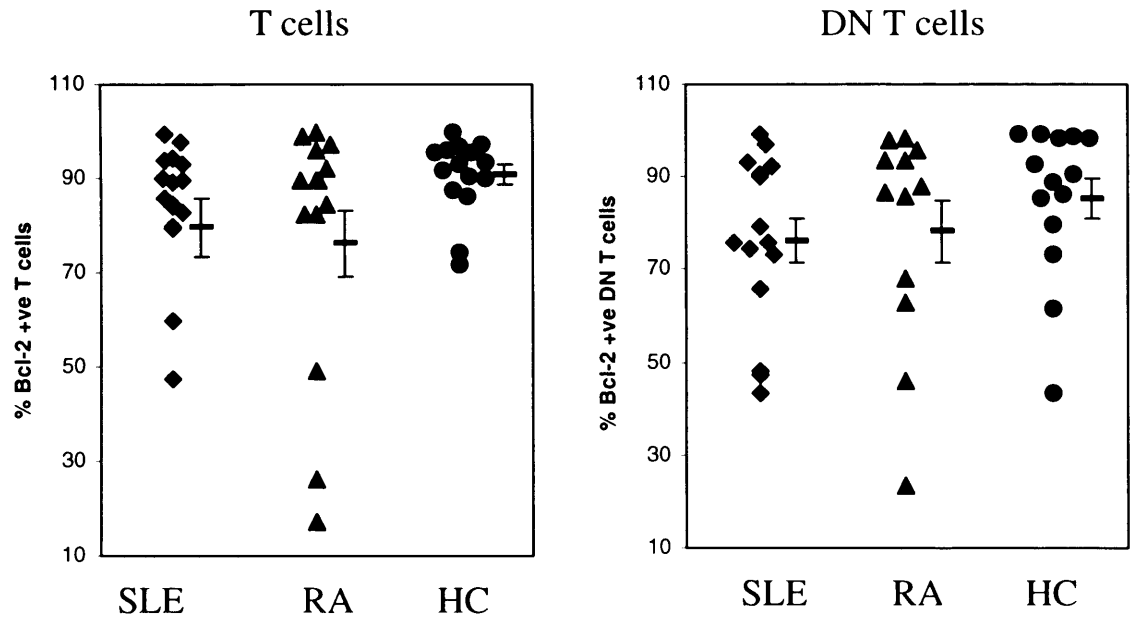


Figure 6.5: Percentages of Bcl-2^+ cells in Patients with SLE (n=14), RA (n=12) and HC (n=14) as determined by intracellular immunofluorescence staining. PBMCs or enriched double negative cells identified by staining with antibodies to CD3 (PE), CD4 and CD8 (CyChrome), were examined for intracellular Bcl-2 expression by staining with an anti-Bcl-2 (FITC) antibody.

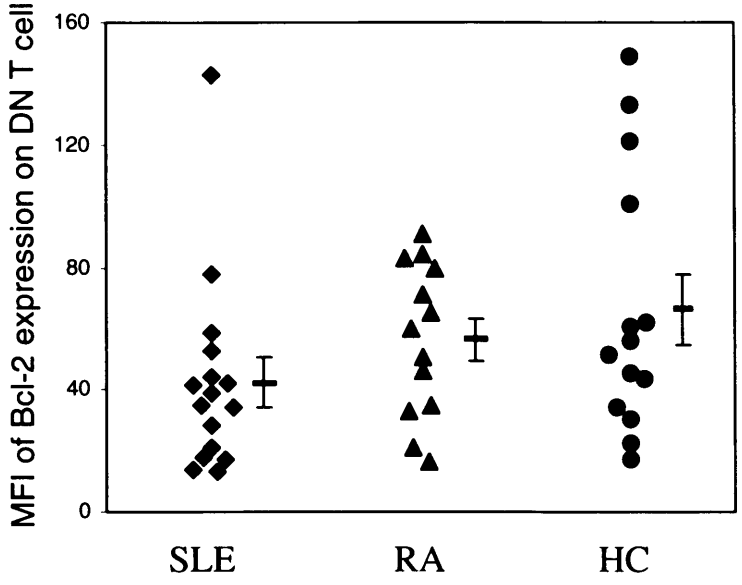


Figure 6.6: Mean Fluorescence intensity (MFI) of Bcl-2 expression determined by flow cytometry, in SLE (n=15), RA (n=13) and HC (n=14) (P> .05) DN T cells. Enriched double negative cells identified by staining with antibodies to CD3 (PE), CD4 and CD8 (CyChrome), were examined for intracellular Bcl-2 expression by staining with an anti-Bcl-2 (FITC) antibody.

6.2.7: Bcl-2/Bax ratio.

One of the apoptotic pathways involves the proteins Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic). In many cell systems, the ratio of Bcl-2 to Bax has been found to regulate apoptosis (Vitali *et al.*, 1992). A higher Bcl-2/Bax ratio indicates cell survival, while a lower ratio is responsible for increased cell death (Aggarwal *et al.*, 1997). Therefore, I examined the expression of Bax and Bcl-2 in DN T cells in culture, over 48 hrs and calculated the ratio of Bcl-2 to Bax expression at time points 0, 24 and 48 hours. DN T cells in culture were harvested at 0, 24 and 48 hrs and intracellular staining for Bcl-2 and Bax proteins were carried out as described in chapter 2. The MFI of expression of each protein was determined by flow cytometry analysis. The ratio of Bcl-2 to Bax MFI was calculated for each population.

The Bcl-2/Bax ratio in the DN T cell population of patients with SLE (2.29±1.32) was significantly higher than that of DN T cells from HC (1.22±0.27)(p=0.02) in freshly isolated

cells (t=0 hrs) as seen in figure 6.7. The $CD4^+/CD8^+$ T cell populations from SLE patients and HC showed no difference in their Bcl-2/Bax ratios (p=0.36).

When DN T cells in culture were harvested at 24 and 48 hours, and the Bcl-2/Bax ratio examined, we could detect no significant differences in ratios between patients with SLE and HC. These results are shown in figure 6.7. As before, the $CD4^+/CD8^+$ T cell population from patients with SLE and HC showed no difference in their Bcl-2/Bax ratio at time points 24 and 48 hours (p>0.05).

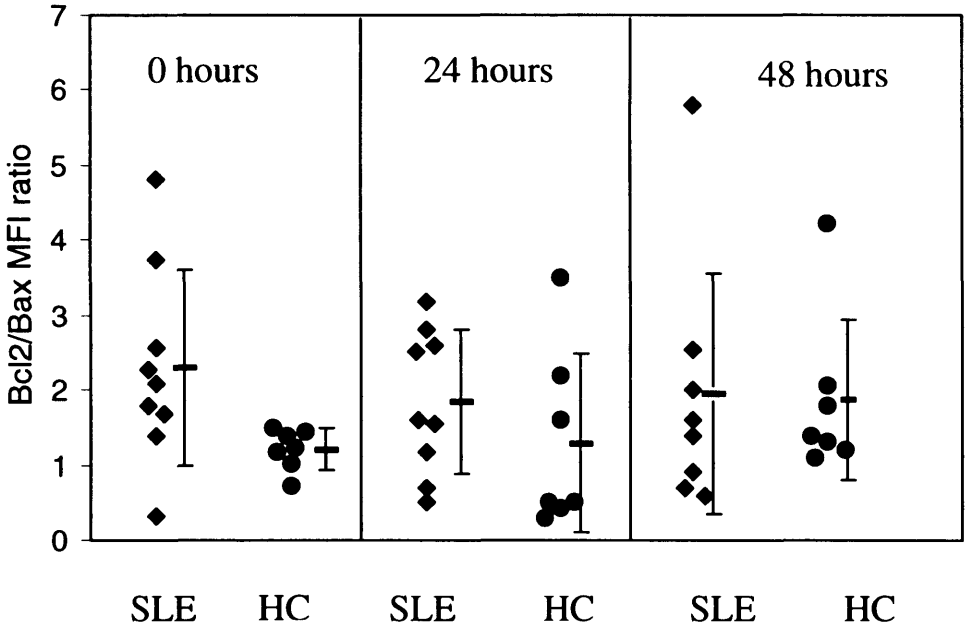


Figure 6.7: Bcl-2/Bax MFI ratios of DN T cells from SLE patients (n=9) and HC (n=7) at time points 0, 24 and 48 hours in culture. Enriched double negative cells identified by staining with antibodies to CD3 (PE), CD4 and CD8 (CYChrome), were examined for intracellular Bcl-2 or Bax expression by staining with anti-Bcl-2 (FITC) or anti-Bax (+ anti-mouse FITC) antibodies.

6.2.8: Bcl-x expression.

The alternatively spliced form of a Bcl-2 homologue, Bcl-x_L, is involved in prolonging survival of cells undergoing apoptosis following various stimuli (Boise *et al.*, 1993). We used an anti-Bcl-x antibody for intracellular staining and FACS analysis of DN T cell cultures at 0, 24 and 48 hours. Immunoblotting studies after anti-Bcl-x immunoprecipitation by Dibbert et al., showed that the Bcl-x expression observed by flow cytometry after immunoflurescence staining was caused solely by Bcl-x_L expression, because no protein from the Bcl-x_S splice form was detected (Dibbert *et al.*, 1998). Therefore, it is possible to conclude that the immunofluoresence staining with the anti-Bcl-x antibody detects the levels of the Bcl-x_L protein.

The intensity (MFI) of Bcl-x expression by freshly isolated (t=0 hrs) DN T cells of patients with SLE (89.6±53.1) was significantly higher than that of HC (45.96±32.23) ($p = 0.03$). On examining Bcl-x levels after 24 hours in culture, DN T cells from patients with SLE (56.4±43.5) showed lower levels of expression than HC (95.01±40.1), though the difference did not reach statistically significant levels ($p = 0.05$). When DN T cells in culture, were harvested at 48 hours and the Bcl-x intensity studied, cells from patients with SLE (68.2±53.1) again showed higher levels of Bcl-x expression compared to HC(39.2±15.7) ($p = 0.04$). These results are shown in figure 6.8. The $CD4^+/CD8^+$ T cell population from patients with SLE and healthy controls showed no difference in Bcl-x expression at 0, 24 or 48 hours ($p > 0.05$) (data not shown).

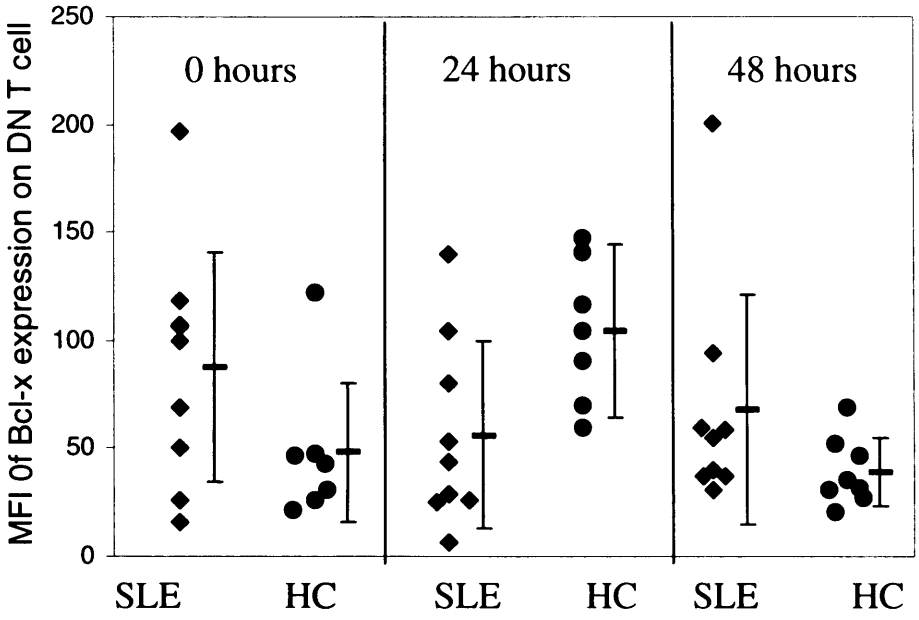


Figure 6.8: Bcl-x expression (MFI) by DN T cells from patients with SLE (n=9) and HC (n=7) at time points 0, 24 and 48 hours in culture. Enriched double negative cells identified by staining with antibodies to CD3 (PE), CD4 and CD8 (CYChrome), were examined for intracellular Bcl-x expression by staining with an anti-Bcl-x antibody, followed by an anti-mouse FITC conjugated secondary antibody.

6.2.9: Summary of results from chapter 6.

The data from this chapter can be summarized as follows:

i. DN T cells showed lower constitutive levels of cell death compared to conventional T cells.

ii. DN T cells from patients with SLE showed even lower levels of spontaneous apoptosis compared to DN T cells of healthy controls. DN T cells from patients with SLE also appeared to be resistant to apoptosis in the first 24 hours in *in vitro* culture.

iii. The expression of Bcl-2 and Fas proteins of the apoptotic pathway was unchanged in DN T cells of patients with SLE compared to healthy controls and patients with RA.

iv. Bcl-2/Bax ratios were significantly higher in DN T cells of patients with SLE when compared to healthy controls in freshly isolated cells.

v. The constitutive expression of Bcl-x anti-apoptotic protein was also significantly higher in DN T cells from patients with SLE when compared to cells from healthy controls.

6.3: Discussion.

A number of studies in both murine models of lupus and human SLE have established an association between the DN T cell population and the disease pathogenesis. Studies have also indicated defective regulation of apoptosis as playing a role in the development of autoimmune diseases such as SLE (Elkon, 1994; McHugh, 2002). Therefore, it was important to study the mechanisms governing cell survival and cell death in the DN T cell population, to understand their contribution to the disease pathogenesis.

6.3.1: Apoptosis in DN T cells.

These studies on the constitutive levels of apoptosis in the DN T cell population have shown significantly lower levels of cell death in these cells compared to conventional ($CD4^+/CD8^+$) T cells. Decreased numbers of apoptotic cells were observed in freshly isolated cells from both patients with SLE and HC. Patients with SLE however, showed fewer numbers of apoptotic DN T cells compared to HC. There were no detectable differences in the numbers of apoptotic cells between patients with SLE and healthy controls in the conventional T cell compartment.

Studies on apoptosis of lymphocytes in SLE reported in literature have revealed contradictory results. Although several studies report abnormalities in the apoptotic process in patients with SLE, other investigators have found normal apoptotic pathways (Aringer *et al.*, 1994; Emlen *et al.*, 1994; Kovacs *et al.*, 1997; McNally *et al.*, 1997; Rose *et al.*, 1997). A more recent study by Caricchio et al., failed to find consistent abnormalities in spontaneous and induced apoptosis in lymphocytes from patients with SLE (Caricchio and Cohen, 1999). The methods used to detect apoptotic cells in the studies mentioned above, were different and might account for the discrepancies between them. Apoptosis was measured in this thesis by annexin-V binding, which reflects phosphatidyl serine exposure and is considered a very early marker of programmed cell death (Pepper *et al.*, 1998). Carrichio et al., also used annexin-V binding as one of the methods of detecting apoptotic cells and the percentages of constitutively apoptotic lymphocytes detected is similar to our results (Caricchio and Cohen, 1999).

The rate of apoptosis in DN T cells of patients with SLE was significantly lower than that of HC in the first 24 hours in culture. By 48 hours, the rates of apoptosis in DN T cells

from both patients and controls reached similar levels. This difference in the rate of apoptosis was unique to the DN T cell population, as conventional T cells from SLE patients and HC showed similar levels of cell death at all time points studied.

Thus, the DN T cells in patients with SLE displayed both lower levels of spontaneous apoptosis and resistance to apoptosis in culture in the first 24 hours.

This resistance to apoptosis of DN T cells in general and those of patients with SLE in particular might provide interesting clues to their role in the immune reaction. One of the roles proposed for DN T cells in the immune system is as suppressor/regulatory cells. Experiments in mice have shown that mature αβ TCR^+ DN T cells possess antigen specific immunoregulatory/ suppressive function and can prevent graft-versus-host disease and autoimmunity (Palathumpat *et al.*, 1992; Abraham *et al.*, 1992; Strober *et al.*, 1996; Priatel *et al.*, 2001). The DN T cells in these studies have been shown to be more resistant to AICD (activation induced cell death) and TCR-cross-linking induced cell death than conventional T cells and are uniquely poised to suppress immune responses and prevent autoimmunity (Yang *et al.*, 1998; Priatel *et al.*, 2001).

6.3.2: Regulation of apoptosis in DN T cells.

In order to explain the resistance of DN T cells from patients with SLE to apoptosis, I studied the expression of pro-and anti- apoptotic proteins Bcl-2, Bax and Bcl-x.

The study results showed no differences in the number of cells expressing Bcl-2 or in the density of expression, in either $CD4^+$/$CD8^+$ T cells or DN T cells of patients with SLE. These results are consistent with reports by Rose et al., studying lymphocytes in a large cohort of patients with SLE (Rose *et al.*, 1995). Expression of Fas was also found unaltered in the DN T cell compartment of patients with SLE when compared to HC, in these studies. These results correlate with investigators reporting that regulation of apoptosis in mature DN T cells in mice do not appear to be influenced by the Fas/FasL or TNF-α/TNFR pathways (Khan *et al.*, 1999). These results however, did not explain the lower constitutive levels of apoptosis and subsequent resistance to cell death in culture, observed in SLE DN T cells.

The pathway of Fas mediated apoptosis has been found to be functionally intact in SLE patients, although increased Fas expression on the surface of B and T cells has been reported, especially in activated lymphocytes (Mysler *et al.*, 1994; Bijl *et al.*, 2001; Bijl *et al.*, 2001). Conversely, studies indicating unchanged Fas expression on SLE lymphocytes have

also been reported (Kovacs *et al.*, 1996). Increased levels of Fas-L and a serum soluble form of Fas (sFas) have also been cited as probable causes for the seemingly increased levels of circulating apoptotic cells in patients with SLE (Kovacs *et al.*, 1997; Rose *et al.*, 1997; Jodo *et al.*, 1997; Bijl *et al.*, 1998).

On examining the Bcl-2/Bax ratio in DN T cells of patients with SLE and controls, I found significantly higher values in SLE DN T cells in freshly isolated cells, consistent with the lower numbers of apoptotic cells observed. After 24 hours incubation in medium, many patients with SLE still showed higher Bcl-2/Bax ratio in their DN T cells compared to HC although, the results were not statistically significant. By 48 hours in culture there were no differences in the Bcl-2/Bax ratios of DN T cells from patients or healthy controls. In these studies, the Bcl-2/Bax ratios in the DN T cells of patients were consistent with the resistance to apoptosis observed in the first 24 hours in culture. Alterations of Bcl-2/Bax ratios are known to regulate apoptosis, thereby determining the survival or death of the cells in which these apoptotic proteins are found (Oltvai *et al.*, 1993). Studies on apoptosis of eosinophils and cord blood lymphocytes have shown that, a high Bcl-2/Bax ratio coupled with high levels of Bcl-x_L promoted cell survival and resistance to apoptosis on activation with mitogens (Aggarwal *et al.*, 1997; Dibbert *et al.*, 1998).

Changes in Bcl-x, expression in DN T cells from patients with SLE over 48 hours in culture were examined. The anti-Bcl-x antibody used in these experiments has been previously shown to mainly detect Bcl-x_L protein expression, because no protein from the Bcl-x_S form could be detected by immunoblotting after immunoprecipitation (Dibbert *et al.*, 1998). Therefore, the Bcl-x protein levels detected in this study were representative mainly of Bcl-x_L protein levels, which protects cells from apoptosis (Boise *et al.*, 1993).

Bcl-x levels in the DN T cells of patients with SLE were significantly greater than HC in freshly isolated cells, although the CD4/ CD8 T cell populations showed no differences in expression. Studies on the regulation of apoptosis in mature DN T cells in mice have shown that these cells constitutively express a higher level of Bcl-x_L, but not Bcl-2, which may contribute to resistance to apoptosis in these cells (Khan *et al.*, 1999). Recent studies by Low et al., have shown that a decrease in levels of Bcl-x and Bcl-2 are required for membrane association of Bax and the subsequent cascade of events leading to apoptosis in cytokine dependent cells (Low *et al.*, 2001). This requirement seems particularly true for apoptosis induced by cytokine withdrawal such as IL-3 removal, whereas cytotoxic drug induced

apoptosis pathways are not affected (Low *et al.*, 2001). These studies contribute towards explaining my observations of an increase in Bax expression accompanied by decreased Bcl-2 and Bcl-x expression after 24 hours in culture, of DN T cells from patients with SLE. An increased level of cell death at 48 hours post-culture followed these changes in the expression levels of proteins of the apoptotic pathway. The absence of cytokines required to support the survival of the patient DN T cells in the culture medium may account for the initiation of apoptosis. The cytokine- IL-3 is a good candidate for this role, as previous studies on both murine and human DN T cells, have reported their unique proliferative response to direct administration of IL-3 (Albert *et al.*, 1998) (Kubota *et al.*, 1992).

6.4: Concluding remarks.

The data in this chapter highlights the low levels of spontaneous apoptosis in DN T cells of patients with SLE and their resistance to apoptosis in culture. The high Bcl-2/Bax ratio and higher levels of Bcl-x observed in the DN T cells from patients contribute to explaining their resistance to apoptosis compared to conventional T cells. This resistance to apoptosis might explain the expanded populations of $\alpha\beta$ TCR^+ DN T cells found in patients with SLE.

Chapter 7.

General Discussion.

The work presented in this thesis investigated the involvement of double negative ($CD3^+$ $CD4^-$ $CD8^-$) T cells in the pathogenesis of SLE. The main conclusions of these studies are outlined below and the questions arising from those results are discussed. A tentative hypothesis is proposed, but, as always, more questions come to light. SLE is a complex disease and many avenues of investigation are in progress to understand the factors involved in initiating and maintaining the disease.

My studies on the DN T cell population in patients with SLE, RA and healthy subjects revealed several distinct features in this population of cells in patients with SLE that suggest their participation in the ongoing inflammatory reaction. The results in this thesis show that the $\alpha\beta$ TCR^+ DN T cell population is significantly expanded in patients with SLE compared to RA autoimmune controls and healthy subjects. The DN T cell population in patients with SLE was also found to be activated and greater numbers of cells expressed IL-4 compared to the same population of cells in healthy individuals. In addition, the DN T cell population in SLE patients showed a greater resistance to apoptosis both *ex-vivo* and *in-vitro*. A higher Bcl-2/Bax ratio and increased expression of Bcl-x anti-apoptotic protein was also observed in the DN T cells of patients with SLE compared to cells from healthy individuals.

7.1: T cells in SLE.

In this study no increase in the numbers of the conventional $CD3^+$ T population in patients with SLE compared to patients with RA and HC were found. Although autoantibody production in SLE has been firmly established as a T cell dependent process, studies on the numbers of $CD3^+$ T cell in patients with SLE have been inconclusive with some groups reporting an increase and others a decrease compared to healthy controls (Erkeller-Yusel *et al.*, 1993) (Glinski *et al.*, 1976; Smolen *et al.*, 1982)). The differences in the patient cohorts studied by the various groups might account for the difference in results.

These studies also did not show an increase in the numbers of $\gamma\delta$ TCR expressing T cells either in the conventional ($CD8^+$), or DN T cell compartments. T cells expressing the $\gamma\delta$ TCR and belonging to either the $CD8^+$or DN T cell compartment have been previously implicated in the disease pathogenesis of SLE and a variety of other autoimmune disorders

(Brennan *et al.*, 1989; Gerli *et al.*, 1991). Their exact role in SLE however, has not been clearly established, with contradictory reports of expanded populations or unchanged or decreased numbers reported in the peripheral blood of patients (Gerli *et al.*, 1991; Spinozzi *et al.*, 1995; Robak *et al.*, 1999; Robak *et al.*, 2001 ; Riccieri *et al.*, 2000). Reports of increased numbers of γδ TCR^+ T lymphocytes in the skin of patients with SLE and displaying a positive correlation with disease activity, could indicate a role for this population at local sites of inflammation and explain the contradictory results reagrding their numbers in peripheral blood (Robak *et al.*,2001).

7.2: DN T cells in SLE.

Examination of the total DN (αβ and γδ TCR^+) in the peripheral blood of patients with SLE showed no significant differences between patients with SLE and autoimmune (RA) or healthy controls. A few studies have previously reported expanded populations of DN T cells in patients with SLE where, treatment with corticosteroids and cyclophosphamide reduced the numbers to normal levels (Lacki *et al.*, 1997; Devi *et al.*, 1998). A few of the patients in our study did show highly expanded (20-35%) populations of DN T cells although there was no correlation with disease activity. Differences in the cohort of patients recruited into the various studies might account for the differences in the numbers of DN T cell populations measured. In murine models of lupus (*lpr*, *gld*) DN T cells have been firmly associated with the disease process with massive accumulation of cells in the lymphoid organs accompanied by nephritis (Reimann, 1991). The role of DN T cells in the pathogenesis of human SLE is however, less defined.

In these studies, the relative numbers of the αβ TCR and γδTCR expressing subpopulations of DN T cell in SLE were examined. Here, significantly expanded populations of αβ TCR^+ DN T cells compared with conventional T cells, in patients with SLE were found which suggested their involvement in the ongoing autoimmune reaction. These results are consistent with studies by other groups describing expanded populations of αβ TCR^+ DN T cells in patients with SLE (Shivakumar *et al.*, 1989; Devi *et al.*, 1998; Sieling *et al.*, 2000). The significance and exact role of αβ TCR^+DN T cells in SLE is yet to be defined. Studies showing expanded populations of αβ TCR^+DN T cells in a number of autoimmune conditions

including, autoimmune lymphoproliferative disorder (ALPS) and systemic sclerosis implicate their participation in autoimmune reactions (Shivakumar *et al.*, 1989; Furukawa, 1997; Illum *et al.*, 1991; Sakamoto *et al.*, 1992; Sieling *et al.*, 2000; Liu *et al.*, 1998). Isolation of αβ TCR^+ and γδ TCR^+ DN T cells with helper function which augment the production of pathogenic anti-DNA autoantibodies in an MHC-unrestricted manner have further suggested a role for these cells in the pathogenesis of SLE (Shivakumar *et al.*, 1989; Rajagopalan *et al.*, 1990). Recent studies also show that SLE DN T cells provide help for IgG production in a CD1 (CD1c) restricted manner(Sieling *et al.*, 2000).

In addition, the numbers of natural killer T cells (NKT), a subset of the DN T cell population and described as TCR $V\alpha24^+$ $V\beta11^+$ have been found to be selectively reduced in a number of autoimmune diseases including systemic sclerosis (SSc), rheumatoid arthritis (RA), insulin-dependent diabetes mellitus (IDDM) and SLE (Dellabona *et al.*, 1994; Sumida *et al.*, 1995; Sumida *et al.*, 1998; Maeda *et al.*, 1999; Wilson *et al.*, 1998).

The role of αβ TCR^+DN T cells in the pathogenesis of SLE can be understood in part by studying the role of this population in the normal immune system. In healthy subjects αβ TCR^+ DN T cells have been described as having a Th0 cytokine profile, both cytotoxic and helper T cell activity, and responding to the hematopoietic stem cell growth factor IL-3 (Kubota *et al.*, 1992) (Groh *et al.*, 1989; Brooks *et al.*, 1990) (Matsumoto *et al.*, 1991) (Porcelli *et al.*, 1992) (Katsikis *et al.*, 1995; Niehues *et al.*, 1999). Based on studies in murine models of transplantation tolerance, experimental autoimmune encephalomyelitis and lymphoproliferative (lpr) mice, a regulatory role has also been advanced for the αβ TCR^+ DN T cells by specifically eliminating syngeneic $CD4^+$ and $CD8^+$ T cells that share the same TCR specificity (Young *et al.*, 2002 ; Young and Zhang, 2002; Carroll, 2001; Ford *et al.*, 2002; Zhang *et al.*, 2001). A regulatory role for IL-4 producing NKT cells (a sub population of αβ TCR^+DN T cells), is further supported by studies showing that the selective reduction of this population is essential for the generation of some autoimmune diseases (Kojo *et al.*, 2001).

Thus, recent reports in the literature suggest that αβ TCR $^+$DN T cells in addition to their potential role as T helper cells contributing to autoantibody production in lupus, could have the contradictory role of regulatory /suppressor T cells involved in containing the disease. The precise role of αβ TCR^+ DN T cell population in the pathogenesis of autoimmune diseases is as yet difficult to define, as long term clonal proliferation of this population of cells

has also been observed in apparently healthy subjects without any associated history of severe illness (Kusunoki *et al.*, 1992).

7.3: Activation status and cytokine profile of SLE DN T cells.

Cell populations taking part in (auto) immune responses would be expected to be 'activated'. The data in this thesis indicates an activated phenotype for DN T cells from patients with SLE, with greater numbers of cells expressing the activation markers HLA-DR, CTLA-4 and CD28. The DN T cells from patients with SLE were also more resistant to *in vitro* stimulation by PHA, perhaps due to their being already activated *in vivo.* The activation status of these SLE DN T cells points to their participation in the ongoing autoimmune reaction.

It has become evident that cell activation events are involved in the deletion of auto-reactive lymphocytes (through induction of apoptosis), and in differentiation of immunoregulatory cells, phenomena that clearly participate in the pathogenesis of autoimmunity. It is therefore feasible that abnormalities in cell activation events could significantly contribute to the immunoregulatory aberrations observed in SLE.

Studies by Siefken et al, have shown that at least *in vitro,* CD28-mediated activation of human T cells without costimulation of the CD3/TCR complex is possible, resulting in antigen-independent activation and expansion of T cells (Siefken *et al.,* 1997). This pathway could contribute to the expansion and activation of increased numbers of $CD28^+$ DN T cells in patients with SLE, which lack the CD4 and CD8 coreceptor for antigen binding through the T cell receptor. Recent studies by Tang et al., have shown that ligation of CD28 molecule partially protected T cell death, likely through the upregulation of the anti-apoptotic protein Bcl-x_L (Tang *et al.*, 2003). As discussed in section 7.4, the studies on SLE DN T cells showed greater numbers of cells were resistant to apoptosis, accompanied by increased expression of Bcl-x. Thus, it might be hypothesised that expression of CD28 by greater numbers of DN T cells from patients with SLE might contribute to the observed resistance in cell death in these cells. In addition, participation of the DN T cells in a cognate interaction with APCs via CD28 might be expected to contribute increased resistance to apoptosis.

The negatively signalling molecule CTLA-4 is involved in establishing and maintaining peripheral T cell tolerance, which controls T cell activation and reactivity. Its attenuating action helps prevent inappropriate initiation of T cell responses to self-antigens

and to terminate ongoing T cell responses. Studies in patients with autoimmune diseases such as SLE, Hashimoto's thyroditis, Grave's diseaseand Insulin dependent diabetes (IDDM), have shown that the CTLA-4 gene could confer disease susceptibilty (Pullmann *et al.*, 1999). In addition, studies in autoimmune (SLE) disease prone mice have shown that treatment with CTLA-4Ig can suppress the pathogenesis of disease and increase longevity by blocking CTLA-4 dependent costimulatory signals (Chu *et al.*, 1996; Wang *et al.*, 2002; Mihara *et al.*, 2000). These studies confirm the involvement of CTLA-4^+ T cells in SLE disease pathogenesis and also suggest the participation of the increased numbers of CTLA-4^+ DN T cells in the disease process.

A greater proportion of SLE DN T cells were also found to be $CD45RA^+$(antigen naïve) compared to DN T cells from HC. One explanation for this observation could be the recruitment of naïve/resting DN T cells to the ongoing immune reaction to either contribute as helper T cells, or contain the autoimmune reaction as regulatory cells. Consistent with our results, studies following CD45 expression and disease progression in SLE T cells have shown the recruitment of naïve/resting T ($CD45RA^+$) cells in active SLE, rather than the reactivation of existing memory ($CD45R0^+$) clones (Gordon *et al.*,1996). Previous studies have shown that mice homozygous for the FasL (*gld/gld*) mutation cannot initiate apoptosis via the Fas/FasL pathway and develop an autoimmune disease characterized by the accumulation of $CD4^-/CD8^-$ DN T cells and a progressive T cell anergy. These DN T cells expressed a high-molecular-weight isoform of CD45 (B220), the CD45RA (Brooks *et al.*, 2001). When a FasL (*gld/gld*) mouse strain with only one functional CD45 allele (CD45(+/-), FasL(*gld/gld*) was produced in order to explore the role that CD45 plays in the lymphoaccumulation and proliferative capacity of the DN T cells, the mice displayed a 10-fold reduction in the DN T cell population and had decreased levels of anti-DNA antibodies and total serum Ig.. These data indicate that CD45 is essential to the accumulation of DN T cells in FasL (*gld/gld*) mice and implicate CD45 as a component of the process of deletion that normally governs the composition of the T cell population. Thus, the increased numbers of $CD45RA^+$ DN T cells in patients with SLE might contribute to the presence of expanded populations of 'activated' $\alpha\beta$ TCR^+ DN T cells shown by my studies.

The data in this thesis has also shown that the $\alpha\beta$ TCR^+ DN T cell compartment of patients with SLE contains more cells positive for intracellular IL-4 than the equivalent cells

of RA autoimmue controls or HC. Recently, a regulatory/immunosupressive role has been suggested for IL-4 producing TCRVα24$^+$ Vβ11$^+$ NK T cells (a sub population of $\alpha\beta^+$ DN T cells) in various autoimmune disorders including SLE (Kojo *et al.*, 2001). Studies on double negative suppressor T cell clones in mice have also shown that they are resistant to apoptosis in the presence of exogenous IL-4 due to the upregulation of Bcl-x_L anti-apoptotic protein (Khan *et al.*, 1999). The greater numbers of IL-4$^+\alpha\beta$ TCR$^+$DN T cells observed in patients with SLE might explain my observations of increased resistance to apoptosis and increased Bcl-x expression in these cells. The IL-4 produced by the cells might act in an autocrine or paracrine manner in prolonging the survival of these cells *in vivo*. Previous studies on the cytokine profile of $\alpha\beta$ TCR$^+$ DN T cells in healthy subjects have also revealed that they are capable of producing IL-4 and IL-10 thus indicating a potential regulatory function (Katsikis *et al.*, 1995). It is possible that the DN T cell population is in fact, a heterogeneous pool of cells comprising compartments with distinct functions.

The antigen specificity of DN T cells in patients with SLE is unknown at present. However, DN T cells have recently been shown to respond to non-peptide antigens presented by the CD1 family of antigen presenting molecules (Porcelli *et al.*, 1992). Unlike MHC antigen presentation, expression of CD4 or CD8 co-receptors or not essential to recognise antigens presented by this family of antigen presentation molecules. A well-defined autoantigen resposible for disease initiation and progression in SLE has yet to be identified. Nuclear material and phospholipids from apoptotic cell debris which escaped the normal clearence process are some of the candidates suggested for the role of autoantigens in SLE, leading to the production of anti-dsDNA and and anti-phospholipid autoantibodies associated with the disease. There is evidence to suggest that these unsual antigens might be presented by the CD1 family of antigen presentation molecules. CD1-restricted T cells (particularly CD1d restricted cells) have been shown to be potent stimulators of humoural immunity, indicating their potential to participate in an autoantibody based autoimmune disease such as SLE (Schofield *et al.*, 1999). It has been proposed that due to infection or trauma, the increased levels of apoptosis or defective clearance of apoptotic material seen in autoimmune diseases such as SLE, can result in apoptotic fragments taken up and presented to T cells by CD1$^+$ dendritic cells (Albert *et al.*, 1998). These apoptotic fragments express cellular phospholipids and antigens normally sequestered from the immune system and might activate T cells (DN T cells?) that recognise them and set an autoimmune response in motion. Identification of T cells

reacting to nucleosomal autoepitopes, and CD1c restricted T cells (perhaps recognising cardiolipin and other self-glycolipids), in patients with SLE lend support to this theory (Shi *et al.*, 1998; Sieling *et al.*, 2000). A regulatory role has also been proposed for CD1-restricted T cells, especially in autoimmune disease conditions. The pattern emerging from studies examining a regulatory role for CD1-restricted DN T cells is that IL-4 production from these cells (especially the NKT cells) protects from self-recognition and that IFN-γ production is associated with disease (Kojo *et al.*, 2001).

7.4: DN T cells in SLE: Apoptosis

In order to understand the mechanisms by which αβ TCR^+ DN T cells accumulate in patients with SLE compared to healthy individuals, the process of apoptosis was studied. Lower levels of constitutive apoptosis in DN T cells of patients with SLE compared to autoimmune controls (RA) and healthy subjects were found. DN T cells in general, showed decreased numbers of apoptotic cells compared to the conventional (CD4 and CD8) compartment, although this phenomenon was more pronounced in SLE DN T cells. When DN T cells from SLE patients and healthy controls were cultured in, a lower rate of apoptosis was observed in SLE DN T cells at 24 hours. By 48 hours numbers of apoptotic cells in DN T cultures from both patients and controls were similar. The change in resistance to apoptosis in SLE DN T cells by 48 hours in culture might be due to absence of anti-apoptotic cytokines which maintain their survival *in vivo*. In fact, studies by Graninger et al., have shown that addition of cytokines such as IL-2, IL-4, IL-7 and IL-15 to *in vitro* cultures of SLE PBMC significantly reduced cell death rates (Graninger *et al.*, 2000). Studies on DN T cells in mice have also shown that they are highly resistant to TCR crosslinking induced apoptosis in the presence of exogenous IL-4 (Khan *et al.*, 1999).

The difference in the numbers of apoptotic cells in freshly isolated and cultured DN T cells, was not observed in the conventional (CD4 and CD8) T cell compartment of patients and controls. Although this result is in contradiction with some published data (Aringer *et al.*, 1994; Emlen *et al.*, 1994; Kovacs *et al.*, 1997) reporting a higher incidence of apoptosis in T cells from patients with SLE it is in agreement with a more recent and comprehensive study by Caricchio et al, who failed to find consistent abnormalities in spontaneous and induced

apoptosis in lymphocytes from SLE patients (Caricchio and Cohen, 1999). Annexin V binding was used by the authors to identify apoptotic cells and the resulting numbers were similar to our results using the same technique (Caricchio and Cohen, 1999). It is not clear from these results whether the increased resistance to apoptosis seen in SLE DN T cells is a contributory cause, or a consequence of the disease process.

To understand the mechanism of resistance to apoptosis of DN T cells further, particularly those from SLE patients, I studied the expression of some pro- and anti- apoptotic proteins involved in the apoptotic process (Fas, Bcl-2, Bcl-x and Bax) in the DN T cell compartment of both patients with SLE and HC. I could detect no differences in the levels of expression of Bcl-2 and Fas proteins in both the $CD4^+/CD8^+$ and DN T cell compartments of patients with SLE, RA and HC in contrast to some previous reports (Graninger, 1992; Gatenby and Irvine, 1994; Aringer *et al.*, 1994). However, data has also been published which support my observations (Chan *et al.*, 1997; Rose *et al.*, 1995; Rose *et al.*, 1997). Differences in the techniques used to detect Fas and Bcl-2 such as western blot analysis and flow cytometry and the disease activity of the patients studied, could account for differences in the results between my studies and others.

Further examination of the Bcl-2/Bax ratios in the DN T cells showed significantly higher values in freshly isolated DN T cells from patients with SLE compared to HC, which would explain the lower numbers of apoptotic cells detected. In addition, the expression of Bcl-x an anti-apoptotic protein, was significantly higher in DN T cells from patients with SLE compared to DN T cells from healthy subjects. The anti-Bcl-x antibody used in our studies has been previously shown to detect mainly the levels of Bcl-x_L in immunofluorescence staining of cells (Dibbert *et al.*, 1998).

Thus, the resistance to apoptosis of DN T cells from patients with SLE could be conferred by the increased expression of Bcl-x_L (anti-apoptotic) and decreased levels of Bax (pro-apoptotic) in these cells, although expression of Bcl-2 is unchanged compared to DN T cells from HC. Previous studies on DN T cells in mice have shown that they constitutively express high levels of BCL-x_L but not Bcl-2 and Bcl-x_L is further up-regulated following TCR-cross-linking and IL-4 stimulation (Khan *et al.*, 1999). Thus, the dysregulation of cytokine profile observed in patients with SLE could perhaps contribute to the prolonged survival of DN T cells by influencing the expression of pro- and anti-apoptotic proteins.

7.5: The role of Double Negative T cells in SLE: friends or foe?

My data together with other published data suggests a role for DN T cells, especially cells expressing the αβ TCR in the pathogenesis of SLE. The results in this thesis indicate the presence of an expanded population of αβ TCR^+ DN T cells, with an activated phenotype, expression of intracellular IL-4 and resistance to apoptosis in the peripheral blood of patients with SLE. These results further support the participation of DN T cells in the autoimmune reaction in patients with SLE. The data in this thesis however, does not answer how these cells could participate in the autoimmune reaction. Two possibilities are:

a) DN T cells have a T helper function and augment the pathogenic autoantibody production in patients with SLE, or

b) DN T cells function as regulatory /supressor cells that act to contain the ongoing autoimmune reaction.

Evidence exists in the literature supporting both these possibilities and is as follows:

a) DN T cells as Helper T cells: Helper cell function has been identified in DN T cells from healthy subjects and DN T cells providing help for the production of pathogenic autoantibodies have been isolated from patients with SLE (Shivakumar *et al.*, 1989; Rajagopalan *et al.*, 1990; Sieling *et al.*, 2000). DN T cells recognising nucleosomal and lipid autoepitopes have also been identified in patients with SLE (Shi *et al.*, 1998; Sieling *et al.*, 2000).

b) DN T cells as regulatory T cells: DN T cells in SLE resemble in many ways the recently described $CD4^+$ $CD25^+$ regulatory T cells-- presenting a highly activated phenotype ($HLA\text{-}DR^+$, $CTLA\text{-}4^+$), apoptosis resistance and expressing higher levels of intracellular IL-4 (Horwitz *et al.*, 2002). Recently, a population of extrathymically derived αβ TCR^+ DN T cells with regulatory functions from the mouse female genital tract have also been reported (Johansson *et al.*, 2003). Reduction in the numbers of $V\alpha24^+$ $V\beta11^+$ NKT cells, defined as a regulatory T cell subset is associated with the occurrence of autoimmune diseases such as systemic sclerosis, SLE, RA and insulin-dependent diabetes mellitus (IDDM) (Kojo *et al.*, 2001). Recently, it has also been suggested that the role of regulatory cells in general immunity may be two-sided, with both autoimmune effector cell function important in tissue maintenance, and regulatory cell function to control this autoimmunity from reaching disease proportions (Schwartz and Kipnis, 2002).

At the start of this project, the available information suggested the possible participation of DN T cells as T helper cells in augmenting the production of pathogenic autoantibodies by B cells, in patients with SLE. The wealth of recently published data now also suggests a potential regulatory/suppressor role for these cells, perhaps attempting to contain the autoimmune reaction. Based on these theories and our results, I propose a model illustrating the possible roles of the DN T cell population to the pathogenesis of SLE.

7.6: Model for the participation of DN T cells in SLE disease pathogenesis.

Figure 7.1 shows possible ways in which DN T cells could contribute to the pathogenesis of SLE.

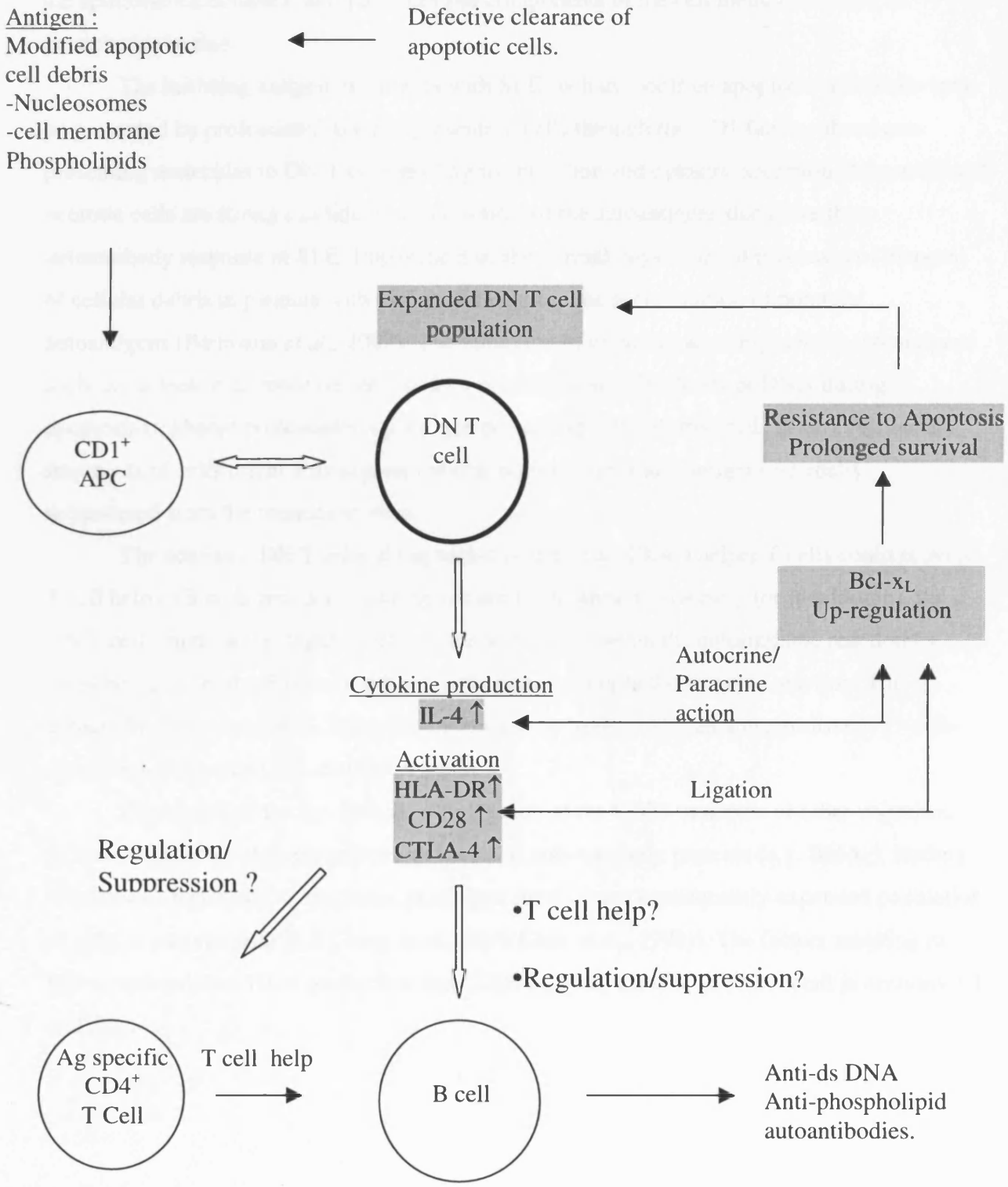


Figure 7.1: Proposed model describing the possible ways of DN T cell participation in SLE disease pathogenesis. Shaded areas denote results described in this thesis.

SLE is characterized by a spectrum of autoantibodies whose respective antigens are abundant in every cell of the body and include chromatin, ribonucleoprotein components of the spliceosome complex, and phospholipid components of the cell membrane, such as phosphatidylserine.

The initiating antigen in patients with SLE such as modified apoptotic cell debris may be presented by professional antigen presenting cells through the CD1 family of antigen presenting molecules to DN T cells leading to activation and cytokine secretion. Apoptotic and necrotic cells are strong candidates as the source of the autoantigens that drive the autoantibody response in SLE. Impairment of the normal physiological processes of disposal of cellular debris in patients with SLE could result in the accumulation of potential autoantigens (Baumann *et al.*, 2002). The induction of of an immune response to autoantigens such as nucleosomes could be enhanced by modifications of histones or DNA during apoptosis or altered presentation by antigen presenting cells (Burns *et al.*, 2000). Apoptotic fragments of cells might also express cellular phospholipids and antigens normally sequestered from the immune system.

The activated DN T cells along with conventional ($CD4^+$) helper T cells could provide T cell help to B cells producing pathogenic autoantibodies. Conversely (or in addition), the DN T cells might act as regulatory/suppressor cells to contain the autoimmune reaction by influencing either the B cells or other T cells participating in the immune reaction. The autoantibody response in SLE has been shown to be mediated by an antigen-driven, T –cell-dependent mechanism in a number of studies.

Expression of the cytokine IL-4 or ligation of the CD28 molecule or other unknown factors might cause the upregulation of levels of anti-apoptotic proteins (e.g. Bcl-x_L), leading to increased resistance to apoptosis, prolonged survival and consequently expanded population of cells in patients with SLE (Tang *et al.*, 2003;Khan *et al.*, 1999)). The factors resulting in Bcl-x_L upregulation (IL-4 production and CD28 ligation) are discussed in detail in sections 7.4 and 7.3.

7.6.1: *Regulatory /Suppressor role of DN T cells.*

Both recently published literature and the studies presented in this thesis on DN T cells in patients with SLE, suggest a regulatory role for all or a subset of the DN T cell population. Extensive studies indicating their regulatory role have been reported in mouse models of transplantation, autoimmune diseases (SLE, EAE, IDDM, SSc) and in normal mice where they are thought to suppress/regulate the function of conventional ($CD4^+/CD8^+$) T cells and B cells (Zhang *et al.*, 2001, Johansson *et al.*, 2003, Kojo *et al.*, 2001). Figure 7.2 illustrates some of the proposed mechanisms by which DN T cells and a subset of these, the NKT cells, are thought to mediate their regulatory activity. Studies of these cells have indicated that they mediate their regulatory function through rapid production of high levels of immunoregulatory cytokines such as IL-4, IFN-γ, TNF and IL-10 (Godfrey *et al.*, 2000). In mouse models of transplantation and in the *lpr* or *gld* mice it has been shown that cell-cell contact is necessary for regulation of target cell activity by DN T cells (Young and Zhang, 2002, Hamad *et al.*, 2003). Studies on NKT cells have indicated that they mediate their regulatory function through rapid production of high levels of immunoregulatory cytokines such as IL-4, IFN-γ, TNF and IL-10 (Godfrey *et al.*, 2000). The regulation of levels of Th1/Th2 cytokines by NKT cells (especially those that are CD1d restricted) cells has been proposed to be significant in reducing inflammation and restricting the proliferation of $CD1d^+$ marginal zone B cells (that are enriched in autoreactive cells) in mouse models of SLE, EAE (experimental autoimmune encephalomyelitis) and IDDM (Godfrey *et al.*, 2000, Chatenoud, 2002).

Thus DN T cells have the potential to regulate all the components of an autoimmune process including the CD8+ T cells involved in inflammation, CD4+T cells that provide help for autoantibody producing B cells and the autoreactive B cells themselves within the splenic marginal zone areas.

My studies on patients with SLE have shown expanded populations of DN T cells, with an activated phenotype, a large percentage of which constitutively produces IL-4 and are resistant to apoptosis. These results, in the context of recently published studies characterizing DN T cells indicate a similar potential immunoregulatory role for this population in patients with SLE. Expansion of the apoptosis-resistant DN T cell population could be a consequence of the disease process aimed at regulating the B cells and conventional T cells participating in the autoimmune response. Further studies on cytokine production by DN T cells from patients

with SLE (IL-10, TGF-β, IFN-γ) and functional studies are needed to verify their precise role in the autoimmune disease process.

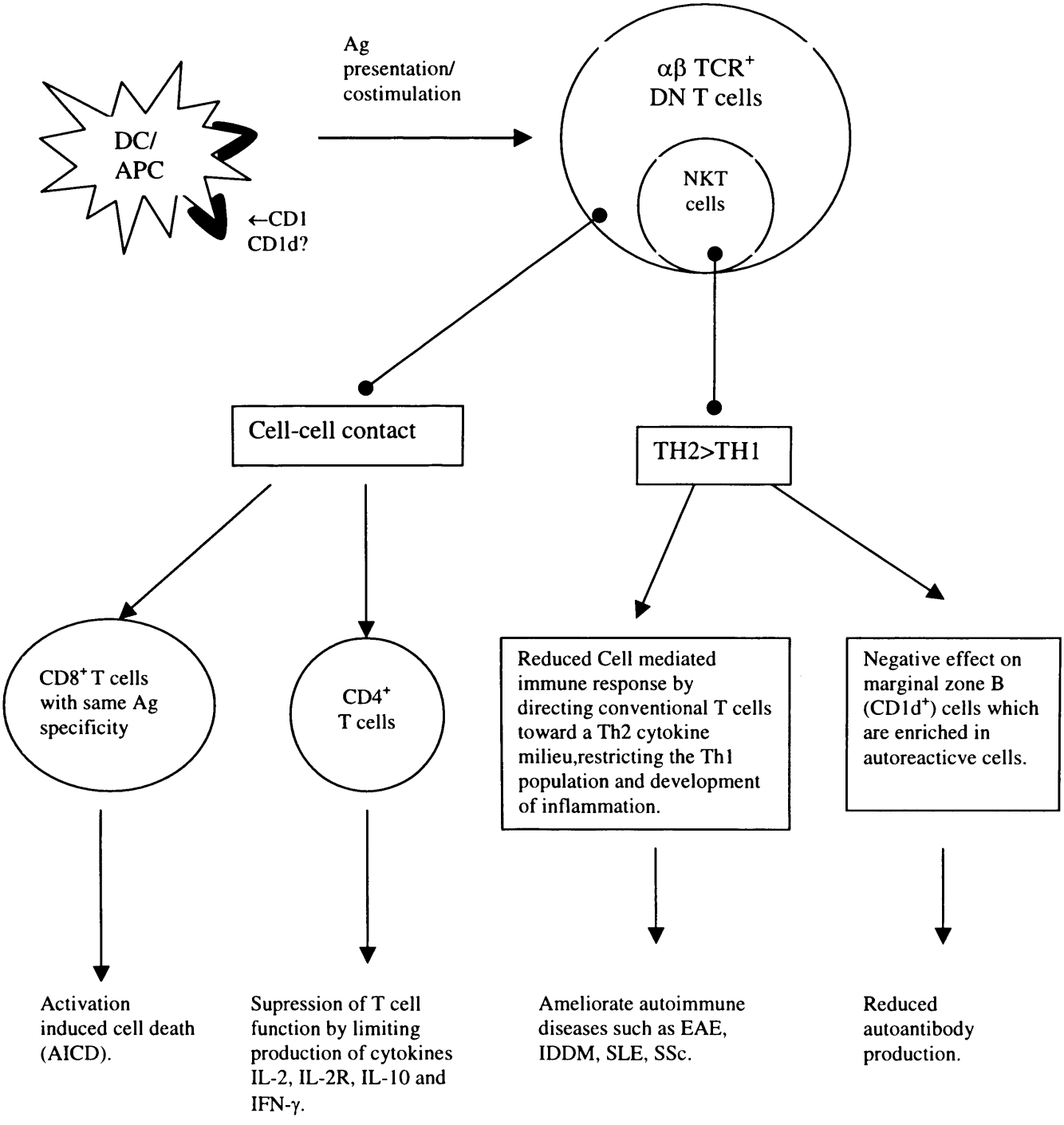


Figure 7.2: Model for the possible roles of DN T cells in autoimmune diseases.

7.7: Future studies

The work in this thesis raises a number of further questions about the role of DN T cells; both as a distinct cell population and their role in the pathogenesis of SLE .

Although, the exact functional contribution of DN T cells to SLE disease pathogenesis has not been defined here, the data presented suggest that they are involved in the process. The work in this thesis shows the importance of the αβ TCR^+ DN T cells in patients with SLE. However, to understand the complete picture of how they contribute to the pathogenesis, their frequency in the tissues of the various organ systems affected in each patient, needs to be evaluated (ideally in untreated patients). This presents practical difficulties since SLE patients do not often undergo biopsies of organs (other than kidney or skin) prior to commencement of treatment.

Presentation of potential SLE autoantigens such as nucleosomes and phospholipids by the CD1 family of antigen presenting molecules to DN T cells needs to be examined in detail. This should provide clues to the circumstances surrounding the initiation of anti-ds DNA and other autoantibody production in lupus. The response by DN T cells to antigen presented by CD1 in terms of activation, cytokine profile and proliferation also requires an in-depth study to further understand their significance in the immune system.

Reference List

Abo, T., Ohteki, T., Seki, S., Koyamada, N., Yoshikai, Y., Masuda, T., Rikiishi, H., and Kumagai, K. (1991). The appearance of T cells bearing self-reactive T cell receptor in the livers of mice injected with bacteria. *J.Exp.Med.* **174**, 417-424.

Abraham, V.S., Sachs, D.H., and Sykes, M. (1992). Mechanism of protection from graft-versus-host disease mortality by IL-2. III. Early reductions in donor T cell subsets and expansion of a CD3+CD4-CD8- cell population. *J.Immunol.* **148**, 3746-3752.

Adams, J.M. and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science* **281**, 1322-1326.

Adams, S., Zordan, T., Sainis, K., and Datta, S. (1990). T cell receptor V beta genes expressed by IgG anti-DNA autoantibody- inducing T cells in lupus nephritis: forbidden receptors and double- negative T cells. *Eur.J.Immunol.* **20**, 1435-1443.

Afeltra, A., Galeazzi, M., Ferri, G.M., Amoroso, A., De Pita, O., Porzio, F., and Bonomo, L. (1993). Expression of CD69 antigen on synovial fluid T cells in patients with rheumatoid arthritis and other chronic synovitis. *Ann.Rheum.Dis.* **52**, 457-460.

Aggarwal, S., Gupta, A., Nagata, S., and Gupta, S. (1997). Programmed cell death (apoptosis) in cord blood lymphocytes. *J.Clin.Immunol.* **17**, 63-73.

Alarcon-Segovia D, Ruiz-Arguelles A, Fishbein E. (1979). Antibody penetration into living cells. I. Intranuclear immunoglobulin in peripheral blood mononuclear cells in mixed connective tissue disease and systemic lupus erythematosus. *Clin Exp Immunol.* **35**, 364-75

Albert, B.M., Bommhardt, U., Cole, M.S., Tso, J.Y., and Zamoyska, R. (1998). CD3 ligation on immature thymocytes generates antagonist-like signals appropriate for CD8 lineage commitment, independently of T cell receptor specificity. *J.Exp.Med.* **187**, 1249-1260.

Albert, M.L., Sauter, B., and Bhardwaj, N. (1998). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86-89.

Alcocer-Varela, J., Alarcon-Riquelme, M., Laffon, A., Sanchez-Madrid, F., and Alarcon-Segovia, D. (1991). Activation markers on peripheral blood T cells from patients with active or inactive systemic lupus erythematosus. Correlation with proliferative responses and production of IL-2. *J.Autoimmun.* **4**, 935-945.

Allison, J.P. and Krummel, M.F. (1995). The Yin and Yang of T cell costimulation. *Science* **270**, 932-933.

Alvarado-de, I.B., Alcocer-Varela, J., Richaud-Patin, Y., Alarcon-Segovia, D., and Llorente, L. (1998). Differential oncogene and TNF-alpha mRNA expression in bone marrow cells from systemic lupus erythematosus patients. *Scand.J.Immunol.* **48**, 551-556.

Andreassen, K., Bendiksen, S., Kjeldsen, E., Van Ghelue, M., Moens, U., Arnesen, E., and Rekvig, O.P. (2002).T cell autoimmunity to histones and nucleosomes is a latent property of the normal immune system. *Arthritis Rheum.* **46**, 1270-1281.

Aringer, M., Wintersberger, W., Steiner, C.W., Kiener, H., Presterl, E., Jaeger, U., Smolen, J.S., and Graninger, W.B. (1994). High levels of bcl-2 protein in circulating T lymphocytes, but not B lymphocytes, of patients with systemic lupus erythematosus. *Arthritis Rheum.* **37**, 1423-1430.

Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., and Luthra, H.S. (1988). The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **31**, 315-324.

Bachmaier, K., Pummerer, C., Shahinian, A., Ionescu, J., Neu, N., Mak, T.W., and Penninger, J.M. (1996). Induction of autoimmunity in the absence of CD28 costimulation. *J.Immunol.* **157**, 1752-1757.

Barber, E.K., Dasgupta, J.D., Schlossman, S.F., Trevillyan, J.M., and Rudd, C.E. (1989). The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc.Natl.Acad.Sci.U.S.A.* **86**, 3277-3281.

Barclay A.N. (1997). The Leucocyte Antigen *Facts Book.* (2nd ed). Harcourt Brace and company. California. U.S.A.

Barnaba, V. and Sinigaglia, F. (1997). Molecular mimicry and T cell-mediated autoimmune disease. *J.Exp.Med.* **185**, 1529-1531.

Baumann, I., Kolowos, W., Voll, R.E., Manger, B., Gaipl, U., Neuhuber, W.L., Kirchner, T., Kalden, J.R., and Herrmann, M. (2002). Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum.* **46**, 191-201.

Benihoud, K., Bonardelle, D., Bobe, P., and Kiger, N. (1997). MRL/lpr CD4- CD8- and CD8+ T cells, respectively, mediate Fas-dependent and perforin cytotoxic pathways. *Eur.J.Immunol.* **27**, 415-420.

Berden, J.H., Licht, R., Van Bruggen, M.C., and Tax, W.J. (1999). Role of nucleosomes for induction and glomerular binding of autoantibodies in lupus nephritis. *Curr.Opin.Nephrol.Hypertens.* **8**, 299-306.

Bijl, M., Horst, G., Limburg, P.C., and Kallenberg, C.G. (2001). Anti-CD3-induced and anti-Fas-induced apoptosis in systemic lupus erythematosus (SLE). *Clin.Exp.Immunol.* **123**, 127-132.

Bijl, M., Horst, G., Limburg, P.C., and Kallenberg, C.G. (2001). Expression of costimulatory molecules on peripheral blood lymphocytes of patients with systemic lupus erythematosus. *Ann.Rheum.Dis.* **60**, 523-526.

Bijl, M., Horst, G., Limburg, P.C., and Kallenberg, C.G. (2001). Fas expression on peripheral blood lymphocytes in systemic lupus erythematosus (SLE): relation to lymphocyte activation and disease activity. *Lupus.* **10**, 866-872.

Bijl, M., van Lopik, T., Limburg, P.C., Spronk, P.E., Jaegers, S.M., Aarden, L.A., Smeenk, R.J., and Kallenberg, G.G. (1998). Do elevated levels of serum-soluble fas contribute to the persistence of activated lymphocytes in systemic lupus erythematosus? *J.Autoimmun.* **11**, 457-463.

Blomberg, J., Nived, O., Pipkorn, R., Bengtsson, A., Erlinge, D., and Sturfelt, G. (1994). Increased antiretroviral antibody reactivity in sera from a defined population of patients with systemic lupus erythematosus. Correlation with autoantibodies and clinical manifestations. *Arthritis Rheum.* **37**, 57-66.

Bodman-Smith, M.D., Anand, A., Durand, V., Youinou, P.Y., and Lydyard, P.M. (2000). Decreased expression of FcgammaRIII (CD16) by gammadelta T cells in patients with rheumatoid arthritis. *Immunology.* **99**, 498-503.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 597-608.

Brennan, F., Plater-Zyberk, C., Maini, R.N., and Feldmann, M. (1989). Coordinate expansion of 'fetal type' lymphocytes (TCR gamma delta+T and CD5+B) in rheumatoid arthritis and primary Sjogren's syndrome. *Clin.Exp.Immunol.* **77**, 175-178.

Bretscher, P. (1992). The two-signal model of lymphocyte activation twenty-one years later. *Immunol.Today* **13**, 74-76.

Brooks, E.G., Balk, S.P., Aupeix, K., Colonna, M., Strominger, J.L., and Groh-Spies, V. (1993). Human T-cell receptor (TCR) alpha/beta + CD4-CD8- T cells express oligoclonal TCRs, share junctional motifs across TCR V beta-gene families, and phenotypically resemble memory T cells. *Proc.Natl.Acad.Sci.U.S.A.* **90**, 11787-11791.

Brooks, E.G., Wirt, D.P., Goldblum, R.M., Vaidya, S., Asuncion, M.T., Patterson, J.C., Ware, C.F., and Klimpel, G.R. (1990). Double-negative (CD4- CD8-) T cells with an alpha/beta T cell receptor. Non-MHC-restricted cytolytic activity and lymphokine production. *J.Immunol.* **144**, 4507-4512.

Brooks, W.P., Lynes, M.A. (2001). Effects of hemizygous CD45 expression in the autoimmune Fasl(gld/gld) syndrome. *Cell Immunol.* **212**, 24-34.

Bruns, A., Blass, S., Hausdorf, G., Burmester, G.R., and Hiepe, F. (2000). Nucleosomes are major T and B cell autoantigens in systemic lupus erythematosus. *Arthritis Rheum.* **43**, 2307-2315.

Budagyan, V.M., Bulanova, E.G., Sharova, N.I., Nikonova, M.F., Stanislav, M.L., and Yarylin, A.A. (1998). The resistance of activated T-cells from SLE patients to apoptosis induced by human thymic stromal cells. *Immunol.Lett.* **60**, 1-5.

Caricchio, R. and Cohen, P.L. (1999). Spontaneous and induced apoptosis in systemic lupus erythematosus: multiple assays fail to reveal consistent abnormalities. *Cell Immunol.* **198**, 54-60.

Carroll, M. (2001). Innate immunity in the etiopathology of autoimmunity. *Nat.Immunol.* **2**, 1089-1090.

Casciola-Rosen, L.A., Anhalt, G., and Rosen, A. (1994). Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes [see comments]. *J.Exp.Med.* **179**, 1317-1330.

Cervera, R., Khamashta, M.A., Font, J., Sebastiani, G.D., Gil, A., Lavilla, P., Domenech, I., Aydintug, A.O., Jedryka-Goral, A., and de Ramon, E. (1993). Systemic lupus erythematosus: clinical and immunologic patterns of disease expression in a cohort of 1,000 patients. The European Working Party on Systemic Lupus Erythematosus. *Medicine (Baltimore.)* **72**, 113-124.

Chan, E.Y., Ko, S.C., and Lau, C.S. (1997). Increased rate of apoptosis and decreased expression of bcl-2 protein in peripheral blood lymphocytes from patients with active systemic lupus erythematosus. *Asian.Pac.J.Allergy Immunol.* **15**, 3-7.

Chao, D.T., Linette, G.P., Boise, L.H., White, L.S., Thompson, C.B., and Korsmeyer, S.J. (1995). Bcl-XL and Bcl-2 repress a common pathway of cell death. *J.Exp.Med.* **182**, 821-828.

Chatenoud, L. (2002). Do NKT cells control autoimmunity*?. J. Clin. Invest.* **110**, 747-748.

Chen, Y.C., Ye, Y.L., and Chiang, B.L. (1997). Establishment and characterization of cloned CD4- CD8- alphabeta-T cell receptor (TCR)-bearing autoreactive T cells from autoimmune NZB x NZW F1 mice. *Clin.Exp.Immunol.* **108**, 52-57.

Cheng, E.H., Levine, B., Boise, L.H., Thompson, C.B., and Hardwick, J.M. (1996). Bax-independent inhibition of apoptosis by Bcl-XL. *Nature* **379**, 554-556.

Christianson, G.J., Blankenburg, R.L., Duffy, T.M., Panka, D., Roths, J.B., Marshak-Rothstein, A., and Roopenian, D.C. (1996). beta2-microglobulin dependence of the lupus-like autoimmune syndrome of MRL-lpr mice. *J.Immunol.* **156**, 4932-4939.

Chu, E.B., Hobbs, M.V., Wilson, C.B., Romball, C.G., Linsley, P.S., and Weigle, W.O. (1996). Intervention of CD4+ cell subset shifts and autoimmunity in the BXSB mouse by murine CTLA4Ig. *J.Immunol.* **156**, 1262-1268.

Cohen, J.J. (1993). Apoptosis. *Immunol.Today* **14**, 126-130.

Cohen, J.J. (1993). Programmed cell death and apoptosis in lymphocyte development and function. *Chest* **103**, 99S-101S.

Cohen, P.L. (1993). T- and B-cell abnormalities in systemic lupus. *J.Invest.Dermatol.* **100**, 69S-72S.

Cohen, P.L., Caricchio, R., Abraham, V., Camenisch, T.D., Jennette, J.C., Roubey, R.A., Earp, H.S., Matsushima, G., and Reap, E.A. *(2002).* Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. *J.Exp.Med..Jul.1.;196.(1.):135.-40.* **196**, 135-140.

Cohen, P.L. and Eisenberg, R.A. (1992). The lpr and gld genes in systemic autoimmunity: life and death in the Fas lane [published erratum appears in Immunol Today 1993 Feb;14(2):97]. *Immunol.Today* **13**, 427-428.

Courtney, P.A., Crockard, A.D., Williamson, K., McConnell, J., Kennedy, R.J., and Bell, A.L. (1999). Lymphocyte apoptosis in systemic lupus erythematosus: relationships with Fas expression, serum soluble Fas and disease activity. *Lupus.* **8**, 508-513.

Crispin, J.C., Martinez, A., de Pablo, P., Velasquillo, C., and Alcocer-Varela, J. (1998). Participation of the CD69 antigen in the T-cell activation process of patients with systemic lupus erythematosus. *Scand.J.Immunol.* **48**, 196-200.

Datta, S.K. (1998). Production of pathogenic antibodies: cognate interactions between autoimmune T and B cells. *Lupus.* **7**, 591-596.

Datta, S.K., Naparstek, Y., and Schwartz, R.S. (1986). In vitro production of an anti-DNA idiotype by lymphocytes of normal subjects and patients with systemic lupus erythematosus. *Clin.Immunol.Immunopathol.* **38**, 302-318.

Datta, S.K., Patel, H., and Berry, D. (1987). Induction of a cationic shift in IgG anti-DNA autoantibodies. Role of T helper cells with classical and novel phenotypes in three murine models of lupus nephritis. *J.Exp.Med.* **165**, 1252-1268.

Dayal, A.K. and Kammer, G.M. (1996). The T cell enigma in lupus. *Arthritis Rheum.* **39**, 23-33.

de la Hera, A., Toribio, M.L., Marquez, C., and Martinez, C. (1985). Interleukin 2 promotes growth and cytolytic activity in human T3+4-8- thymocytes. *Proc.Natl.Acad.Sci.U.S.A.* **82**, 6268-6271.

Dean, G.S., Tyrrell-Price, J., Crawley, E., and Isenberg, D.A. (2000). Cytokines and systemic lupus erythematosus. *Ann.Rheum.Dis.* **59**, 243-251.

Dejbakhsh-Jones, S., Jerabek, L., Weissman, I.L., and Strober, S. (1995). Extrathymic maturation of alpha beta T cells from hemopoietic stem cells. *J.Immunol.* **155**, 3338-3344.

Dellabona, P., Padovan, E., Casorati, G., Brockhaus, M., and Lanzavecchia, A. (1994). An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J.Exp.Med.* **180**, 1171-1176.

Denfeld, R.W., Kind, P., Sontheimer, R.D., Schopf, E., and Simon, J.C. (1997). In situ expression of B7 and CD28 receptor families in skin lesions of patients with lupus erythematosus. *Arthritis Rheum.* **40**, 814-821.

Desai-Mehta, A., Mao, C., Rajagopalan, S., Robinson, T., and Datta, S.K.(1995). Structure and specificity of T cell receptors expressed by potentially pathogenic anti-DNA autoantibody-inducing T cells in human lupus. *J.Clin.Invest.* **95**, 531-541.

Devi, B.S., Van Noordin, S., Krausz, T., and Davies, K.A. (1998). Peripheral blood lymphocytes in SLE--hyperexpression of CD154 on T and B lymphocytes and increased number of double negative T cells. *J.Autoimmun.* **11**, 471-475.

Dibbert, B., Daigle, I., Braun, D., Schranz, C., Weber, M., Blaser, K., Zangemeister-Wittke, U., Akbar, A.N., and Simon, H.U. (1998). Role for Bcl-xL in delayed eosinophil apoptosis mediated by granulocyte-macrophage colony-stimulating factor and interleukin-5. *Blood* **92**, 778-783.

Dziarski, R. (1988). Autoimmunity: Polyclonal activation or antigen induction?. *Immunology Today*. **9**, 340.

Elkon, K.B. (1994). Apoptosis in SLE--too little or too much? *Clin.Exp.Rheumatol.* **12**, 553-559.

Emlen W., Niebur J., and Kadera R. (1994). Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. *J.Immunol.* **152**, 3685-3692.

Emlen, W., Niebur, J., and Kadera, R. (1994). Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. *J.Immunol.* **152**, 3685-3692.

Erkeller-Yusel, F., Hulstaart, F., Hannet, I., Isenberg, D., and Lydyard, P. (1993). Lymphocyte subsets in a large cohort of patients with systemic lupus erythematosus. *Lupus.* **2**, 227-231.

Esdaile, J.M., Abrahamowicz, M., Joseph, L., MacKenzie, T., Li, Y., and Danoff, D. (1996). Laboratory tests as predictors of disease exacerbations in systemic lupus erythematosus. Why some tests fail. *Arthritis Rheum.* **39**, 370-378.

Fadok, V.A., Bratton, D.L., and Henson, P.M. (2001).Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J.Clin.Invest.* **108**, 957-962.

Falcini, F., Azzari, C., Gelli, V.A., Luchetti, M., Gabrielli, A., Calzolari, A., Pignone, A., Generini, S., and Matucci, C.M. (1999). Reduction of bcl-2 in T cells during immunosuppressive therapy in patients with severe juvenile onset systemic lupus erythematosus. *Clin.Immunol.* **93**, 59-64.

Feng, Y.(1997). [Studies on Fas ligand expression in patients with systemic lupus erythematosus]. *Hokkaido.Igaku.Zasshi.* **72**, 443-455.

Fernandez-Gutierrez, B., de Miguel, S., Morado, C., Hernandez-Garcia, C., Banares, A., and Jover, J.A. (1998). Defective early T and T-dependent B cell activation in systemic lupus erythematosus. *Lupus.* **7**, 314-322.

Fessel, W.J. (1974). Systemic lupus erythematosus in the community. Incidence, prevalence, outcome, and first symptoms; the high prevalence in black women. *Arch.Intern.Med.* **134**, 1027-1035.

Finck, B.K., Chan, B., and Wofsy, D. (1994). Interleukin 6 promotes murine lupus in NZB/NZW F1 mice. *J.Clin.Invest.* **94**, 585-591.

Finck, B.K., Linsley, P.S., and Wofsy, D. (1994). Treatment of murine lupus with CTLA4Ig. *Science* **265**, 1225-1227.

Ford, M.S., Young, K.J., Zhang, Z., Ohashi, P.S., and Zhang, L. (2002). The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. *J.Exp.Med.* **196**, 261-267.

Fowlkes, B.J., Kruisbeek, A.M., Ton-That, H., Weston, M.A., Coligan, J.E., Schwartz, R.H., and Pardoll, D.M. (1987). A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. *Nature* **329**, 251-254.

Fredriksen, K., Skogsholm, A., Flaegstad, T., Traavik, T., and Rekvig, O.P. (1993). Antibodies to dsDNA are produced during primary BK virus infection in man, indicating that anti-dsDNA antibodies may be related to virus replication in vivo. *Scand.J.Immunol.* **38**, 401-406.

Funauchi M., Ikoma S., Enomoto H., and Horiuchi A. (1998). Decreased Th1-like and Increased Th2-like Cells in Systemic Lupus Erythematosus. *Scand. J. Rheumatol.* **27**, 219-224.

Funauchi, M., Yu, H., Sugiyama, M., Ikoma, S., Ohno, M., Kinoshita, K., Hamada, K., and Kanamaru, A. (1999). Increased interleukin-4 production by NK T cells in systemic lupus erythematosus. *Clin.Immunol.* **92**, 197-202.

Furukawa, F. (1997). Animal models of cutaneous lupus erythematosus and lupus erythematosus photosensitivity. *Lupus.* **6**, 193-202.

Fuss, I.J., Strober, W., Dale, J.K., Fritz, S., Pearlstein, G.R., Puck, J.M., Lenardo, M.J., and Straus, S.E. (1997). Characteristic T helper 2 T cell cytokine abnormalities in autoimmune lymphoproliferative syndrome, a syndrome marked by defective apoptosis and humoral autoimmunity. *J.Immunol.* **158**, 1912-1918.

Garcia-Cozar, F.J., Molina, I.J., Cuadrado, M.J., Marubayashi, M., Pena, J., and Santamaria, M.(1996). Defective B7 expression on antigen-presenting cells underlying T cell activation abnormalities in systemic lupus erythematosus (SLE) patients. *Clin.Exp.Immunol.* **104**, 72-79.

Gatenby, P.A. and Irvine, M. (1994). The bcl-2 proto-oncogene is overexpressed in systemic lupus erythematosus. *J.Autoimmun.* **7**, 623-631.

Gavalchin, J. and Datta, S.K. (1987). The NZB X SWR model of lupus nephritis. II. Autoantibodies deposited in renal lesions show a distinctive and restricted idiotypic diversity. *J.Immunol.* **138**, 138-148.

Gerli, R., Agea, E., Bertotto, A., Tognellini, R., Flenghi, L., Spinozzi, F., Velardi, A., and Grignani, F. (1991). Analysis of T cells bearing different isotypic forms of the gamma/delta T cell receptor in patients with systemic autoimmune diseases. *J.Rheumatol.* **18**, 1504-1510.

Giese, T. and Davidson, W.F. (1995). The accumulation of B220+ CD4- CD8- (DN) T cells in C3H-lpr/lpr mice is not accelerated by the stimulation of CD8+ T cells or B220+ DN T cells with staphylococcal enterotoxin B and occurs independently of V beta 8+ T cells. *Int.Immunol.* **7**, 1213-1223.

Gladman, D.D., Goldsmith, C.H., Urowitz, M.B., Bacon, P., Bombardier, C., Isenberg, D., Kalunian, K., Liang, M.H., Maddison, P., and Nived, O. (1992). Crosscultural validation and reliability of 3 disease activity indices in systemic lupus erythematosus. *J.Rheumatol.* **19**, 608-611.

Gladman, D.D., Goldsmith, C.H., Urowitz, M.B., Bacon, P., Bombardier, C., Isenberg, D., Kalunian, K., Liang, M.H., Maddison, P., and Nived, O. (1994). Sensitivity to change of 3 Systemic Lupus Erythematosus Disease Activity Indices: international validation. *J.Rheumatol.* **21**, 1468-1471.

Glinski, W., Gershwin, M.E., Budman, D.R., and Steinberg, A.D. (1976). Study of lymphocyte subpopulations in normal humans and patients with systemic lupus erythematosus by fractionation of peripheral blood lymphocytes on a discontinuous Ficoll gradient. *Clin.Exp.Immunol.* **26**, 228-238.

Godfrey, D.I., Hammond, K., Poulton, L.D., Smyth, M.J., Baxter, A.G. (2000). NKT cells: facts, functions and Fallacies. *Immunol Today.* **21**, 573-583.

Gordon, C., Matthews, N., Schlesinger, B.C., Akbar, A.N., Bacon, P.A., Emery, P., and Salmon, M. (1996). Active systemic lupus erythematosus is associated with the recruitment of naive/resting T cells. *Br.J.Rheumatol.* **35**, 226-230.

Graninger, W.B. (1992). Transcriptional overexpression of the proto-oncogene bcl-2 in patients with systemic Lupus erythematosus. *Wien.Klin.Wochenschr.* **104**, 205-207.

Graninger, W.B., Steiner, C.W., Graninger, M.T., Aringer, M., and Smolen, J.S. (2000). Cytokine regulation of apoptosis and Bcl-2 expression in lymphocytes of patients with systemic lupus erythematosus. *Cell Death.Differ.* **7**, 966-972.

Groen, H., Aslander, M., Bootsma, H., van der Mark, T.W., Kallenberg, C.G., and Postma, D.S.(1993). Bronchoalveolar lavage cell analysis and lung function impairment in patients with systemic lupus erythematosus (SLE). *Clin.Exp.Immunol.* **94**, 127-133.

Groh, V., Fabbi, M., Hochstenbach, F., Maziarz, R.T., and Strominger, J.L.(1989). Double-negative (CD4-CD8-) lymphocytes bearing T-cell receptor alpha and beta chains in normal human skin. *Proc.Natl.Acad.Sci.U.S.A.* **86**, 5059-5063.

Groh, V., Porcelli, S., Fabbi, M., Lanier, L.L., Picker, L.J., Anderson, T., Warnke, R.A., Bhan, A.K., Strominger, J.L., and Brenner, M.B. (1989). Human lymphocytes bearing T cell receptor gamma/delta are phenotypically diverse and evenly distributed throughout the lymphoid system. *J.Exp.Med.* **169**, 1277-1294.

Hahn, B.H.(1998). Antibodies to DNA. *N.Engl.J.Med.* **338**, 1359-1368.

Hahn, B.H. and Ebling, F.M.(1984). A public idiotypic determinant is present on spontaneous cationic IgG antibodies to DNA from mice of unrelated lupus-prone strains. *J.Immunol.* **133**, 3015-3019.

Hamad, A., Mohamood, A., Trujillo, C., Huang, C.T., Yuan, E., Schneck, J.P. (2003). $B220^+$ Double-negative T cells suppress polyclonal T cell activation by a Fas-independent mechanism that involves inhibition of IL-2 production. *J.Immunol.* **171**, 2421-2426.

Hardgrave, K.L., Neas, B.R., Scofield, R.H., and Harley, J.B. (1993). Antibodies to vesicular stomatitis virus proteins in patients with systemic lupus erythematosus and in normal subjects. *Arthritis Rheum.* **36**, 962-970.

Haq, I and Isenberg, D.A. (2002). Systemic lupus erythematosus. *Medicine.***30**, 6-12.

Hashimoto, M., Nonaka, S., Furuta, E., Wada, T., Suenaga, Y., Yasuda, M., Shingu, M., and Nobunaga, M. (1994). Methotrexate for steroid-resistant systemic lupus erythematosus. *Clin.Rheumatol.* **13**, 280-283.

Hay, E.M., Bacon, P.A., Gordon, C., Isenberg, D.A., Maddison, P., Snaith, M.L., Symmons, D.P., Viner, N., and Zoma, A. (1993). The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. *Q.J.Med.* **86**, 447-458.

Hayday, A. and Geng, L. (1997). Gamma delta cells regulate autoimmunity. *Curr.Opin.Immunol.* **9**, 884-889.

Heiligenhaus, A., Dutt, J.E., and Foster, C.S. (1996). Histology and immunopathology of systemic lupus erythematosus affecting the conjunctiva. *Eye* **10**, 425-432.

Hernandez-Fuentes, M.P., Reyes, E., Prieto, A., Zea, A., Villa, L., Sanchez-Atrio, A., Esquivel, F., and Alvarez-Mon, M. (1999). Defective proliferative response of T lymphocytes from patients with inactive systemic lupus erythematosus. *J.Rheumatol.* **26**, 1518-1526.

Hernandez-Garcia, C., Fernandez-Gutierrez, B., Morado, I.C., Banares, A.A., and Jover, J.A. (1996). The CD69 activation pathway in rheumatoid arthritis synovial fluid T cells. *Arthritis Rheum.* **39**, 1277-1286.

Herrmann, M., Voll, R.E., Zoller, O.M., Hagenhofer, M., Ponner, B.B., and Kalden, J.R. (1998). Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis Rheum.* **41**, 1241-1250.

Heward, J. and Gough, S.C. (1997). Genetic susceptibility to the development of autoimmune disease. *Clin.Sci.(Lond.)* **93**, 479-491.

Hochberg, M.C. and Petri, M. (1993). Clinical features of systemic lupus erythematosus. *Curr.Opin.Rheumatol.* **5**, 575-586.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D., and Korsmeyer, S.J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**, 334-336.

Hooks, J.J., Jordan, G.W., Cupps, T., Moutsopoulos, H.M., Fauci, A.S., and Notkins, A.L. (1982). Multiple interferons in the circulation of patients with systemic lupus erythematosus and vasculitis. *Arthritis Rheum.* **25**, 396-400.

Horwitz D.A. and Jacob C.O. (1994). The cytokine network in the pathogenesis of systemic lupus erythematosus and possible therapeutic implications. *Springer Semin Immunopathol* **16** , 181-200.

Horwitz, D.A., Stohl, W., Gray, J.D. (1997). *Dubois' Lupus Erythematosus* (D.J.Wallace and B.H Hahn Eds, Ed.) 5Th ed edition. Williams & Wilkins, Baltimore. 154-194.

Horwitz, D.A., Gray, J.D., and Zheng, S.G.(2002). The potential of human regulatory T cells generated ex vivo as a treatment for lupus and other chronic inflammatory diseases. *Arthritis Res.* **4**, 241-246.

Horwitz, D.A., Tang, F.L., Stimmler, M.M., Oki, A., and Gray, J.D. (1997). Decreased T cell response to anti-CD2 in systemic lupus erythematosus and reversal by anti-CD28: evidence for impaired T cell-accessory cell interaction. *Arthritis Rheum.* **40**, 822-833.

Howland, K.C., Ausubel, L.J., London, C.A., and Abbas, A.K. (2000). The roles of CD28 and CD40 ligand in T cell activation and tolerance. *J.Immunol.* **164**, 4465-4470.

Huang, L. and Crispe, I.N. (1992). Distinctive selection mechanisms govern the T cell receptor repertoire of peripheral CD4-CD8- alpha/beta T cells. *J.Exp.Med.* **176**, 699-706.

Illum, N., Ralfkiaer, E., Pallesen, G., and Geisler, C. (1991). Phenotypical and functional characterization of double-negative (CD4- CD8-) alpha beta T-cell receptor positive cells from an immunodeficient patient. *Scand.J.Immunol.* **34**, 635-645.

Inaba, K., Turley, S., Yamaide, F., Iyoda, T., Mahnke, K., Inaba, M., Pack, M., Subklewe, M., Sauter, B., Sheff, D., Albert, M., Bhardwaj, N., Mellman, I., and Steinman, R.M.(1998). Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J.Exp.Med.* **188**, 2163-2173.

Ioannou, Y., Isenberg D.A. (2002). Current concepts for the management of systemic lupus erythematosus in adults: a therapeutic challenge. *Postgrad. Med. J.* **78**, 599-606.

Isenberg D.A. (1993). Systemic Lupus Erythematosus. *Hospital Update* 598-606.

Isenberg, D.A. and Collins, C. (1985). Detection of cross-reactive anti-DNA antibody idiotypes on renal tissue-bound immunoglobulins from lupus patients. *J.Clin.Invest.* **76**, 287-294.

Isenberg D.A. and Horsfall, A.C. (1998). Systemic Lupus Erythematosus. *Oxford Text book of Rheumatology.* (Maddison, P.J., Isenberg, D.A. Glass, D.N. Ed.). 2nd Edn. Oxford University Press. 1145-1180.

Ishikawa, S., Akakura, S., Abe, M., Terashima, K., Chijiiwa, K., Nishimura, H., Hirose, S., and Shirai, T. (1998). A subset of CD4+ T cells expressing early activation antigen CD69 in murine lupus: possible abnormal regulatory role for cytokine imbalance. *J.Immunol.* **161**, 1267-1273.

Itoh, N., Tsujimoto, Y., and Nagata, S. (1993). Effect of bcl-2 on Fas antigen-mediated cell death. *J.Immunol.* **151**, 621-627.

Jacob, C.O., van der Meide, P.H., and McDevitt, H.O. (1987). In vivo treatment of (NZB X NZW)F1 lupus-like nephritis with monoclonal antibody to gamma interferon. *J.Exp.Med.* **166**, 798-803.

Jacobs R, Pawlak CR, Mikeska E, Meyer-Olson D, Martin M, Heijnen CJ, Schedlowski M, Schmidt RE. (2001). Systemic lupus erythematosus and rheumatoid arthritis patients differ from healthy controls in their cytokine pattern after stress exposure. *Rheumatology (Oxford)* **40**, 868-75.

Jayawardena-Wolf, J. and Bendelac, A. (2001). CD1 and lipid antigens: intracellular pathways for antigen presentation. *Curr.Opin.Immunol.* **13**, 109-113.

Jodo, S., Kobayashi, S., Kayagaki, N., Ogura, N., Feng, Y., Amasaki, Y., Fujisaku, A., Azuma, M., Yagita, H., Okumura, K., and Koike, T. (1997). Serum levels of soluble Fas/APO-1 (CD95) and its molecular structure in patients with systemic lupus erythematosus (SLE) and other autoimmune diseases. *Clin.Exp.Immunol.* **107**, 89-95.

Johansson, M. and Lycke, N. (2003). A Unique Population of Extrathymically Derived alphabetaTCR(+)CD4(-)CD8(-) T Cells with Regulatory Functions Dominates the Mouse Female Genital Tract. *J Immunol.* **170**,1659-66.

June, C.H., Bluestone, J.A., Nadler, L.M., and Thompson, C.B. (1994). The B7 and CD28 receptor families. *Immunol.Today* **15**, 321-331.

Kaliyaperumal, A., Michaels, M.A., and Datta, S.K. (2002). Naturally processed chromatin peptides reveal a major autoepitope that primes pathogenic T and B cells of lupus. *J.Immunol.* **168**, 2530-2537.

Kammer, G.M. (1999). High prevalence of T cell type I protein kinase A deficiency in systemic lupus erythematosus. *Arthritis Rheum.* **42**, 1458-1465.

Kammer, G.M., Tsokos, G.C. (1998). Emerging concepts of the molecular basis for estrogen effects on T lymphocytes in systemic lupus erythematosus. *Clin. Immunol.Immunopathol.* **89**, 192-195.

Kaneko, H., Nakajima, A., and Azuma, M. (1997). [Manipulation of costimulatory pathways in autoimmune disease]. *Nippon.Rinsho.* **55**, 1531-1536.

Kaneko, H., Saito, K., Hashimoto, H., Yagita, H., Okumura, K., and Azuma, M. (1996). Preferential elimination of CD28+ T cells in systemic lupus erythematosus (SLE) and the relation with activation-induced apoptosis. *Clin.Exp.Immunol.* **106**, 218-229.

Kanemitsu, S., Takabayashi, A., Sasaki, Y., Kuromaru, R., Ihara, K., Kaku, Y., Sakai, K., and Hara, T. (1999). Association of interleukin-4 receptor and interleukin-4 promoter gene polymorphisms with systemic lupus erythematosus. *Arthritis Rheum.* **42**, 1298-1300.

Katsikis, P.D., Cohen, S.B., Murison, J.G., Uren, J., Hibbart, L.M., Callard, R.E., Di Padova, F., Feldmann, M., and Londei, M. (1995). Human alpha beta T-cell receptor CD4-CD8 T-cell clones are predominantly Th0-like. *Immunology* **84**, 501-504.

Kelley W.N. (1990). *Textbook of Rheumatology* (Kelley W.N., R.S.H.E.D., Ed.) W.B. Saunders and Company, Philadelphia. 1015-1027.

Khan, Q., Penninger, J.M., Yang, L., Marra, L.E., Kozieradzki, I., and Zhang, L. (1999). Regulation of apoptosis in mature alphabeta+CD4-CD8- antigen

-specific suppressor T cell clones. *J.Immunol.* **162**, 5860-5867.

Kirou, K.A. and Crow, M.K. (1999). New pieces to the SLE cytokine puzzle. *Clin.Immunol.* **91**, 1-5.

Klinman, D.M. and Steinberg, A.D. (1987). Systemic autoimmune disease arises from polyclonal B cell activation. *J.Exp.Med.* **165**, 1755-1760.

Koffler, D., Carr, R., Agnello, V., Thoburn, R., and Kunkel, H.G. (1971). Antibodies to polynucleotides in human sera: antigenic specificity and relation to disease. *J.Exp.Med.* **134**, 294-312.

Kojo, S., Adachi, Y., Keino, H., Taniguchi, M., and Sumida, T. (2001). Dysfunction of T cell receptor AV24AJ18+, BV11+ double-negative regulatory natural killer T cells in autoimmune diseases. *Arthritis Rheum.* **44**, 1127-1138.

Kotzin, B.L. (1996). Systemic lupus erythematosus. *Cell.* **85**, 303-306.

Kovacs, B., Liossis, S.N., Dennis, G.J., and Tsokos, G.C.(1997). Increased expression of functional Fas-ligand in activated T cells from patients with systemic lupus erythematosus. *Autoimmunity.* **25**, 213-221.

Kovacs, B., Vassilopoulos, D., Vogelgesang, S.A., and Tsokos, G.C.(1996). Defective CD3-mediated cell death in activated T cells from patients with systemic lupus erythematosus: role of decreased intracellular TNF- alpha. *Clin.Immunol.Immunopathol.* **81**, 293-302.

Kristiansen, O.P., Larsen, Z.M., and Pociot, F. (2000). CTLA-4 in autoimmune diseases--a general susceptibility gene to autoimmunity? *Genes Immun.* **1**, 170-184.

Kubota, H., Okazaki, H., Onuma, M., Kano, S., Hattori, M., and Minato, N. (1992). CD3+4-8- alpha beta T cell population with biased T cell receptor V gene usage. Presence in bone marrow and possible involvement of IL-3 for their extrathymic development. *J.Immunol.* **149**, 1143-1150.

Kuby J. (1997). *Immunology* . (3rd ed). W.H. Freeman and Company, NewYork.

Kusunoki, Y., Hirai, Y., Kyoizumi, S., and Akiyama, M. (1992). Evidence for in vivo clonal proliferation of unique population of blood CD4-/CD8- T cells bearing T-cell receptor alpha and beta chains in two normal men. *Blood* **79**, 2965-2972.

Lacki, J.K., Mackiewicz, S.H., Leszczynski, P., and Muller, W. (1997). The effect of intravenous cyclophosphamide pulse on peripheral blood lymphocytes in lupus erythematosus patients. *Rheumatol.Int.* **17**, 55-60.

Lahita, R.G. (1999). The role of Sex hormones in systemic lupus erythematosus. *Curr. Opin. Rheumatol.* **11**, 352-360.

Lahita, R., Kluger, J., Drayer, D.E., Koffler, D., and Reidenberg, M.M.(1979). Antibodies to nuclear antigens in patients treated with procainamide or acetylprocainamide. *N.Engl.J.Med.* **301**, 1382-1385.

Lenschow, D.J., Walunas, T.L., and Bluestone, J.A.(1996). CD28/B7 system of T cell costimulation. *Annu.Rev.Immunol.* **14:233-58**, 233-258.

Linker-Israeli M., Deans R.J., Wallace D.J., Prehn J., Ozeri-Chen T., and Klinenberg J.R.(1991). Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. *J.Immunol.* **147**, 117-123.

Linker-Israeli, M., Honda, M., Nand, R., Mandyam, R., Mengesha, E., Wallace, D.J., Metzger, A., Beharier, B., and Klinenberg, J.R.(1999). Exogenous IL-10 and IL-4 down-regulate IL-6 production by SLE-derived PBMC. *Clin.Immunol.* **91**, 6-16.

Linker-Israeli, M., Quismorio, F.P.J., and Horwitz, D.A.(1990). CD8+ lymphocytes from patients with systemic lupus erythematosus sustain, rather than suppress, spontaneous polyclonal IgG production and synergize with CD4+ cells to support autoantibody synthesis. *Arthritis Rheum.* **33**, 1216-1225.

Liu, M.F., Li, J.S., Weng, T.H., and Lei, H.Y.(1998). Double-negative (CD4-CD8-) TCRalphabeta+ cells in patients with systemic lupus erythematosus. *Scand.J.Rheumatol.* **27**, 130-134.

Liu, M.F., Liu, H.S., Wang, C.R., and Lei, H.Y.(1998). Expression of CTLA-4 molecule in peripheral blood T lymphocytes from patients with systemic lupus erythematosus. *J.Clin.Immunol.* **18**, 392-398.

Lohmann, C.M., League, A.A., Clark, W.S., Lawson, D., DeRose, P.B., and Cohen, C. (2000). Bcl-2: bax and bcl-2: Bcl-x ratios by image cytometric quantitation of immunohistochemical expression in ovarian carcinoma: correlation with prognosis. *Cytometry.* **42**, 61-66.

Londei, M., Verhoef, A., De Berardinis, P., Kissonerghis, M., Grubeck-Loebenstein, B., and Feldmann, M. (1989). Definition of a population of CD4-8- T cells that express the alpha beta T-cell receptor and respond to interleukins 2, 3, and 4. *Proc.Natl.Acad.Sci.* **86**, 8502-8506.

Lorenz, H.M., Grunke, M., Hieronymus, T., Herrmann, M., Kuhnel, A., Manger, B., and Kalden, J.R. (1997). In vitro apoptosis and expression of apoptosis-related molecules in lymphocytes from patients with systemic lupus erythematosus and other autoimmune diseases [see comments]. *Arthritis Rheum.* **40**, 306-317.

Low, W., Olmos-Centenera, G., Madsen, C., Leverrier, Y., and Collins, M.K. (2001). Role of Bax in apoptosis of IL-3-dependent cells. *Oncogene.* **20**, 4476-4483.

Lu, L., Kaliyaperumal, A., Boumpas, D.T., and Datta, S.K. (1999). Major peptide autoepitopes for nucleosome-specific T cells of human lupus. *J.Clin.Invest.* **104**, 345-355.

Maas, K., Chan, S., Parker, J., Slater, A., Moore, J., Olsen, N., and Aune, T.M. (2002). Cutting edge: molecular portrait of human autoimmune disease. *J.Immunol.* **169**, 5-9.

Maeda, T., Keino, H., Asahara, H., Taniguchi, M., Nishioka, K., and Sumida, T. (1999). Decreased TCR AV24AJ18+ double-negative T cells in rheumatoid synovium [letter]. *Rheumatology* **38**, 186-188.

Marilyn, A., Slsky, M.D. (2002). Newtherapies in systemic lupus erythematosus. *Best. pract. Res. Clinic. Rheumatol.*. **16**, 293-312

Marzio, R., Mauel, J., and Betz-Corradin, S. (1999). CD69 and regulation of the immune function. *Immunopharmacol.Immunotoxicol.* **21**, 565-582.

Mason, L.J. and Isenberg, D.A. (1998). Immunopathogenesis of SLE. *Baillieres.Clin.Rheumatol.* **12**, 385-403.

Matsumoto, M., Yasukawa, M., Inatsuki, A., and Kobayashi, Y. (1991). Human double-negative (CD4-CD8-) T cells bearing alpha beta T cell receptor possess both helper and cytotoxic activities. *Clin.Exp.Immunol.* **85**, 525-530.

McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., McKearn, J.P., and Korsmeyer, S.J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell.* **57**, 79-88.

McHugh, N.J. (2002). Systemic lupus erythematosus and dysregulated apoptosis-what is the evidence? *Rheumatology.* **41**, 242-245.

McNally, J., Yoo, D.H., Drappa, J., Chu, J.L., Yagita, H., Friedman, S.M., and Elkon, K.B.(1997). Fas ligand expression and function in systemic lupus erythematosus. *J.Immunol.* **159**, 4628-4636.

Mevorach, D.(1999). The immune response to apoptotic cells. *Ann.N.Y.Acad.Sci.* **887**, 191-198.

Mihara, M., Tan, I., Chuzhin, Y., Reddy, B., Budhai, L., Holzer, A., Gu, Y., and Davidson, A. (2000). CTLA4Ig inhibits T cell-dependent B-cell maturation in murine systemic lupus erythematosus. *J.Clin.Invest.* **106**, 91-101.

Miret, C., Font, J., Molina, R., Garcia-Carrasco, M., Filella, X., Ramos, M., Ballesta, R.C., and Ingelmo, M. (1999). BCL-2 oncogene (B cell lymphoma/leukemia-2) levels correlate with systemic lupus erythematosus disease activity. *Anticancer Res.* **19**, 3073-3076.

Molina, J.F., Citera, G., Rosler, D., Cuellar, M.L., Molina, J., Felipe, O., and Espinoza, L.R. (1995). Coexistence of human immunodeficiency virus infection and systemic lupus erythematosus. *J.Rheumatol.* **22**, 347-350.

Monneaux F, Muller S. (2001). Epitope spreading in systemic lupus erythematosus: identification of triggering peptide sequences. *Arthritis Rheum.* **46**(6),1430-8.

Mosmann TR, Sad S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today.* **17,**138-46.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* . **136**, 2348-57.

Murison, J.G., Quaratino, S., Kahan, M., Verhoef, A., and Londei, M. (1993). Definition of unique traits of human CD4-CD8- alpha beta T cells. *Clin.Exp.Immunol.* **93**, 464-470.

Mysler, E., Bini, P., Drappa, J., Ramos, P., Friedman, S.M., Krammer, P.H., and Elkon, K.B.(1994). The apoptosis-1/Fas protein in human systemic lupus erythematosus. *J.Clin.Invest.* **93**, 1029-1034.

Nagata, S. and Golstein, P.(1995). The Fas death factor. *Science* **267**, 1449-1456.

Nagy, G., Pallinger, E., Antal-Szalmas, P., Aleksza, M., Marschalko, M., Brozik, M., Falus, A., and Gergely, P. (2000). Measurement of intracellular interferon-gamma and interleukin-4 in whole blood T lymphocytes from patients with systemic lupus erythematosus. *Immunol.Lett.* **74**, 207-210.

Nakajima, A., Hirose, S., Yagita, H., and Okumura, K. (1997). Roles of IL-4 and IL-12 in the development of lupus in NZB/W F1 mice. *J.Immunol.* **158**, 1466-1472.

Nazareth, M., Fanti, P., Schwach, C., Poppenberg, K., Janis, K., and Aronica, S.M. (2001). Altered Bax expression and decreased apoptosis in bone marrow cells of lupus-susceptible NZB/W mice. *Lupus.* **10**, 785-793.

Neidhart, M., Pataki, F., Michel, B.A., and Fehr, K. (1996). CD45 isoforms expression on CD4+ and CD8+ peripheral blood T- lymphocytes is related to auto-immune processes and hematological manifestations in systemic lupus erythematosus. *Schweiz.Med.Wochenschr.* **126**, 1922-1925.

Niehues, T., Eichelbauer, D., and Schneider, E.M. (1999). Functional characteristics of human peripheral blood alpha/betaTCR+, CD4- and CD8- double-negative (DN) T cells. *Microbiol.Immunol.* **43**, 153-159.

Niehues, T., Gulwani-Akolkar, B., Akolkar, P.N., Tax, W., and Silver, J. (1994). Unique phenotype and distinct TCR V beta repertoire in human peripheral blood alpha beta TCR+, CD4-, and CD8- double negative T cells. *J.Immunol.* **152**, 1072-1081.

Ohsako, S., Hara, M., Harigai, M., Fukasawa, C., and Kashiwazaki, S. (1994). Expression and function of Fas antigen and bcl-2 in human systemic lupus erythematosus lymphocytes. *Clin.Immunol.Immunopathol.* **73**, 109-114.

Oishi, Y., Sakamoto, A., Kurasawa, K., Nakajima, H., Nakao, A., Nakagawa, N., Tanabe, E., Saito, Y., and Iwamoto, I. *(2000).* CD4-CD8- T cells bearing invariant Valpha24JalphaQ TCR alpha-chain are decreased in patients with atopic diseases. *Clin.Exp.Immunol.* **119**, 404-411.

Oishi, Y., Sumida, T., Sakamoto, A., Kita, Y., Kurasawa, K., Nawata, Y., Takabayashi, K., Takahashi, H., Yoshida, S., Taniguchi, M., Saito, Y., and Iwamoto, I. (2001). Selective reduction and recovery of invariant Valpha24JalphaQ T cell receptor T cells in correlation with disease activity in patients with systemic lupus erythematosus. *J.Rheumatol.* **28**, 275-283.

Okuyama, R., Abo, T., Seki, S., Ohteki, T., Sugiura, K., Kusumi, A., and Kumagai, K. (1992). Estrogen administration activates extrathymic T cell differentiation in the liver. *J.Exp.Med.* **175**, 661-669.

Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619.

Osorio, L.M., Jondal, M., and Aguilar-Santelises, M. (1998). Regulation of B-CLL apoptosis through membrane receptors and Bcl-2 family proteins. *Leuk.Lymphoma.* **30**, 247-256.

Pala, P., Hussell, T., Openshaw, P. J. (2000). Flow cytometric measurement of intracellular cytokines. *J Immunol Methods.* **243**, 107-24.

Palathumpat, V., Dejbakhsh-Jones, S., Holm, B., and Strober, S. (1992). Different subsets of T cells in the adult mouse bone marrow and spleen induce or suppress acute graft-versus-host disease. *J.Immunol.* **149**, 808-817.

Palathumpat, V., Dejbakhsh-Jones, S., Holm, B., Wang, H., Liang, O., and Strober, S. (1992). Studies of CD4- CD8- alpha beta bone marrow T cells with suppressor activity. *J.Immunol.* **148**, 373-380.

Parnes, J.R.(1989). Molecular biology and function of CD4 and CD8. *Adv.Immunol.* **44,** 265-311.

Pelkonen, J. and Palacios, R.(1990). LD1: a CD4-CD8- TCR alpha beta/CD3+ peripheral T cell line with helper function for B lymphocytes. *Int.Immunol.* **2**, 555-562.

Pepper, C., Thomas, A., Tucker, H., Hoy, T., and Bentley, P. (1998). Flow cytometric assessment of three different methods for the measurement of in vitro apoptosis. *Leuk.Res.* **22** , 439-444.

Perniok, A., Wedekind, F., Herrmann, M., Specker, C., and Schneider, M. (1998). High levels of circulating early apoptic peripheral blood mononuclear cells in systemic lupus erythematosus. *Lupus.* **7**, 113-118.

Petri, M., Genovese, M., Engle, E., and Hochberg, M. (1991). Definition, incidence, and clinical description of flare in systemic lupus erythematosus. A prospective cohort study. *Arthritis Rheum.* **34**, 937-944.

Pisetsky, D.S., Jelinek, D.F., McAnally, L.M., Reich, C.F., and Lipsky, P.E. (1990). In vitro autoantibody production by normal adult and cord blood B cells. *J.Clin.Invest.* **85**, 899-903.

Plana, M., Font, J., Vinas, O., Martorell, J., Ingelmo, M., and Vives, J. (1994). Responsiveness of T lymphocytes from systemic lupus erythematosus to signals provided through CD26 antigen. *Clin.Immunol.Immunopathol.* **72**, 227-232.

Porcelli, S., Morita, C.T., and Brenner, M.B. (1992). CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. *Nature* **360**, 593-597.

Portales-Perez, D., Gonzalez-Amaro, R., Abud-Mendoza, C., and Sanchez-Armass, S. (1997). Abnormalities in CD69 expression, cytosolic pH and Ca2+ during activation of lymphocytes from patients with systemic lupus erythematosus. *Lupus.* **6**, 48-56.

Priatel, J.J., Utting, O., and Teh, H.S. (2001). TCR/self-antigen interactions drive double-negative T cell peripheral expansion and differentiation into suppressor cells. *J.Immunol.* **167**, 6188-6194.

Prud'Homme, G.J., Bocarro, D.C., and Luke, E.C. (1991). Clonal deletion and autoreactivity in extrathymic CD4-CD8-(double negative) T cell receptor-alpha/beta T cells. *J.Immunol.* **147**, 3314-3318.

Pullmann, R.J., Lukac, J., Skerenova, M., Rovensky, J., Hybenova, J., Melus, V., Celec, S., Pullmann, R., and Hyrdel, R. (1999). Cytotoxic T lymphocyte antigen 4 (CTLA-4) dimorphism in patients with systemic lupus erythematosus [In Process Citation]. *Clin.Exp.Rheumatol.* **17**, 725-729.

Radic, M.Z. and Weigert, M. (1994). Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu.Rev.Immunol.* **12**, 487-520.

Rajagopalan, S., Zordan, T., Tsokos, G.C., and Datta, S.K. (1990). Pathogenic anti-DNA autoantibody-inducing T helper cell lines from patients with active lupus nephritis: isolation of CD4-8- T helper cell lines that express the gamma delta T-cell antigen receptor. *Proc.Natl.Acad.Sci.* **87**, 7020-7024.

Ravirajan, C.T., Sarraf, C.E., Anilkumar, T.V., Golding, M.C., Alison, M.R., and Isenberg, D.A. (1996). An analysis of apoptosis in lymphoid organs and lupus disease in murine systemic lupus erythematosus (SLE). *Clin.Exp.Immunol.* **105**, 306-312.

Reed, J.C.(1997). Double identity for proteins of the Bcl-2 family. *Nature* **387**, 773-776.

Reimann, J.(1991). Double-negative (CD4-CD8-), TCR alpha beta-expressing, peripheral T cells [editorial]. *Scand.J.Immunol.* **34**, 679-688.

Reininger, L., Santiago, M.L., Takahashi, S., Fossati, L., and Izui, S.(1996). T helper cell subsets in the pathogenesis of systemic lupus erythematosus. *Ann.Med.Interne.(Paris.)* **147**, 467-471.

Rekvig, O.P., Moens, U., Sundsfjord, A., Bredholt, G., Osei, A., Haaheim, H., Traavik, T., Arnesen, E., and Haga, H.J. (1997). Experimental expression in mice and spontaneous expression in human SLE of polyomavirus T-antigen. A molecular basis for induction of antibodies to DNA and eukaryotic transcription factors. *J.Clin.Invest.* **99**, 2045-2054.

Riccieri, V., Spadaro, A., Parisi, G., Taccari, E., Moretti, T., Bernardini, G., Favaroni, M., and Strom, R. (2000). Down-regulation of natural killer cells and of gamma/delta T cells in systemic lupus erythematosus. Does it correlate to autoimmunity and to laboratory indices of disease activity? *Lupus.* **9**, 333-337.

Richaud-Patin, Y., Alcocer-Varela, J., and Llorente, L. (1995). High levels of TH2 cytokine gene expression in systemic lupus erythematosus. *Rev.Invest.Clin.* **47**, 267-272.

Rider, V. and Abdou, N.I. (2001). Gender differences in autoimmunity: molecular basis for estrogen effects in systemic lupus erythematosus. *Int.Immunopharmacol.* **1**, 1009-1024.

Rider, V., Foster, R.T., Evans, M., Suenaga, R., Abdou, N.I. (1998). Gender differences in autoimmune diseases: estrogen increases calcineurin expression in systemic lupus erythematosus. *Clin. Immunol. Immunopathol.* **89**, 171-180.

Rivas, A., Koide, J., Laus, R., and Engleman, E.G. (1990). Alloantigen-specific cytotoxic clones bearing the alpha,beta T cell antigen receptor but not CD4 or CD8 molecules. *J.Immunol.* **145**, 470-476.

Robak, E., Blonski, J.Z., Bartkowiak, J., Niewiadomska, H., Sysa-Jedrzejowska, A., and Robak, T. (1999). Circulating TCR gammadelta cells in the patients with systemic lupus erythematosus [In Process Citation]. *Mediators.Inflamm.* **8**, 305-312.

Robak, E., Niewiadomska, H., Robak, T., Bartkowiak, J., Blonski, J.Z., Wozniacka, A., Pomorski, L., and Sysa-Jedrezejowska, A. (2001). Lymphocyctes Tgammadelta in clinically normal skin and peripheral blood of patients with systemic lupus erythematosus and their correlation with disease activity. *Mediators.Inflamm.* **10**, 179-189.

Romagnani, S. (1997). The Th1/Th2 paradigm. *Immunol.Today* **18**, 263-266.

Rose, L.M., Latchman, D.S., and Isenberg, D.A. (1995). Bcl-2 expression is unaltered in unfractionated peripheral blood mononuclear cells in patients with systemic lupus erythematosus. *Br.J.Rheumatol.* **34**, 316-320.

Rose, L.M., Latchman, D.S., and Isenberg, D.A. (1997). Apoptosis in peripheral lymphocytes in systemic lupus erythematosus: a review. *Br.J.Rheumatol.* **36**, 158-163.

Rose, L.M., Latchman, D.S., and Isenberg, D.A. (1997). Elevated soluble fas production in SLE correlates with HLA status not with disease activity. *Lupus.* **6**, 717-722.

Rovere, P., Sabbadini, M.G., Fazzini, F., Bondanza, A., Zimmermann, V.S., Rugarli, C., and Manfredi, A.A. (2000). Remnants of suicidal cells fostering systemic autoaggression. Apoptosis in the origin and maintenance of autoimmunity. *Arthritis Rheum.* **43**, 1663-1672.

Rovere, P., Sabbadini, M.G., Vallinoto, C., Fascio, U., Recigno, M., Crosti, M., Ricciardi-Castagnoli, P., Balestrieri, G., Tincani, A., and Manfredi, A.A. (1999). Dendritic cell presentation of antigens from apoptotic cells in a proinflammatory context: role of opsonizing anti-beta2-glycoprotein I antibodies. *Arthritis Rheum.* **42**, 1412-1420.

Rovere, P., Vallinoto, C., Bondanza, A., Crosti, M.C., Rescigno, M., Ricciardi-Castagnoli, P., Rugarli, C., and Manfredi, A.A. (1998). Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J.Immunol.* **161**, 4467-4471.

Rubin, R. (1999). Etilogy and mechanisms of drug-induced lupus. *Curr. Opin. Rhematol.* **11**, 357-369.

Rudensky, A.Y. (1995). Endogenous peptides associated with MHC class II and selection of CD4 T cells. *Semin.Immunol.* **7**, 399-409.

Rulifson, I.C., Sperling, A.I., Fields, P.E., Fitch, F.W., and Bluestone, J.A. (1997). CD28 costimulation promotes the production of Th2 cytokines. *J.Immunol.* **158**, 658-665.

Ryffel, B., Car, B.D., Gunn, H., Roman, D., Hiestand, P., and Mihatsch, M.J. (1994). Interleukin-6 exacerbates glomerulonephritis in (NZB x NZW)F1 mice. *Am.J.Pathol.* **144**, 927-937.

Sakaguchi, S., Fukuma, K., Kuribayashi, K., and Masuda, T. (1985). Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J.Exp.Med.* **161**, 72-87.

Sakamoto, A., Sumida, T., Maeda, T., Itoh, M., Asai, T., Takahashi, H., Yoshida, S., Koike, T., and Tomioka, H. (1992). T cell receptor V beta repertoire of double-negative alpha/beta T cells in patients with systemic sclerosis. *Arthritis Rheum.* **35**, 944-948.

Sakata, K., Sakata, A., Vela-Roch, N., Espinosa, R., Escalante, A., Kong, L., Nakabayashi, T., Cheng, J., Talal, N., and Dang, H. (1998). Fas (CD95)-transduced signal preferentially stimulates lupus peripheral T lymphocytes. *Eur.J.Immunol.* **28**, 2648-2660.

Santiago M.L., Fossati L., Jaquet C., Muller W., Izui S., and Reininger L. (1997). Interleukin-4 Protects Against a Genetically Linked Lupus-like Autoimmune Syndrome. *Journal of Experimental Medicine* **185**, 65-70.

Schaible, U.E. and Kaufmann, S.H. (2000). CD1 molecules and CD1-dependent T cells in bacterial infections: a link from innate to acquired immunity? *Semin.Immunol.* **12**, 527-535.

Scheinecker, C., Zwolfer, B., Koller, M., Manner, G., and Smolen, J.S. (2001). Alterations of dendritic cells in systemic lupus erythematosus: phenotypic and functional deficiencies. *Arthritis Rheum.* **44**, 856-865.

Schiffer, L.E., Hussain, N., Wang, X., Huang, W., Sinha, J., Ramanujam, M., Davidson, A. (2002). Lowering anti-dsDNA antibodies- what's new? *Lupus.* **11**, 885-894.

Schofield, L., McConville, M.J., Hansen, D., Campbell, A.S., Fraser-Reid, B., Grusby, M.J., and Tachado, S.D. (1999). CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. *Science* **283**, 225-229.

Schorlemmer, H.U., Dickneite, G., Kanzy, E.J., and Enssle, K.H. (1995). Modulation of the immunoglobulin dysregulation in GvH- and SLE-like diseases by the murine IL-4 receptor (IL-4-R). *Inflamm.Res.* **44**, S194-S196

Schwarting A., Wada T., Kinoshita M., Tesch G., and Kelley V.R. (1998). IFN-gamma Receptor Signalling is Essential for the Initiation, Acceleration, and Destruction of Autoimmune Kidney Disease in MRL-Fas(lpr) Mice. *Journal of Immunology* **161**, 494-503.

Schwartz, M. and Kipnis, J. (2002). Autoimmunity on alert: naturally occurring regulatory CD4(+)CD25(+) T cells as part of the evolutionary compromise between a 'need' and a 'risk'. *Trends.Immunol.* **23**, 530-534.

Schwartz, R.H. (2003). T cell anergy. *Annu.Rev.Immunol.* **21**, 305-334.

Seddon, B. and Mason, D. (1996). Effects of cytokines and glucocorticoids on endogenous class II MHC antigen expression by activated rat CD4+ T cells. Mature CD4+ CD8- thymocytes are phenotypically heterogeneous on activation. *Int.Immunol.* **8**, 1185-1193.

Seki, M., Ushiyama, C., Seta, N., Abe, K., Fukazawa, T., Asakawa, J., Takasaki, Y., and Hashimoto, H. (1998). Apoptosis of lymphocytes induced by glucocorticoids and relationship to therapeutic efficacy in patients with systemic lupus erythematosus. *Arthritis Rheum.* **41**, 823-830.

Seman, M., Boudaly, S., Roger, T., Morisset, J., and Pham, G. (1990). Autoreactive T cells in normal mice: unrestricted recognition of self peptides on dendritic cell I-A molecules by CD4-CD8- T cell receptor alpha/beta+ T cell clones expressing V beta 8.1 gene segments. *Eur.J.Immunol.* **20**, 1265-1272.

Sfikakis, P.P., Oglesby, R., Sfikakis, P., and Tsokos, G.C. (1994). B7/BB1 provides an important costimulatory signal for CD3-mediated T lymphocyte proliferation in patients with systemic lupus erythematosus (SLE). *Clin.Exp.Immunol.* **96**, 8-14.

Shamshiev, A., Gober, H.J., Donda, A., Mazorra, Z., Mori, L., and De Libero, G. (2002). Presentation of the same glycolipid by different CD1 molecules. *J.Exp.Med.* **195**, 1013-1021.

Shi, Y., Kaliyaperumal, A., Lu, L., Southwood, S., Sette, A., Michaels, M.A., and Datta, S.K. (1998). Promiscuous presentation and recognition of nucleosomal autoepitopes in lupus: role of autoimmune T cell receptor alpha chain. *J.Exp.Med.* **187**, 367-378.

Shivakumar, S., Tsokos, G.C., and Datta, S.K. (1989). T cell receptor alpha/beta expressing double-negative (CD4-/CD8-) and CD4+ T helper cells in humans augment the production of pathogenic anti- DNA autoantibodies associated with lupus nephritis. *J.Immunol.* **143**, 103-112.

Siefken, R., Kurrle, R., Schwinzer, R. (1997). CD28-Mediated activation of resting T cells without costimulation of the CD3/TCR complex. *Cell. Immunol.* **176**, 59-65.

Sieling, P.A. (2000). CD1-Restricted T cells: T cells with a unique immunological niche. *Clin.Immunol.* **96**, 3-10.

Sieling, P.A., Porcelli, S.A., Duong, B.T., Spada, F., Bloom, B.R., Diamond, B., and Hahn, B.H. (2000). Human double-negative T cells in systemic lupus erythematosus provide help for IgG and are restricted by CD1c. *J.Immunol.* **165**, 5338-5344.

Singh, V.K., Mehrotra, S., and Agarwal, S.S. (1999). The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol.Res.* **20**, 147-161.

Smolen, J.S., Chused, T.M., Leiserson, W.M., Reeves, J.P., Alling, D., and Steinberg, A.D. (1982). Heterogeneity of immunoregulatory T-cell subsets in systemic lupus erythematosus. Correlation with clinical features. *Am.J.Med.* **72**, 783-790.

Sobel, E.S., Mohan, C., Morel, L., Schiffenbauer, J., and Wakeland, E.K. (1999). Genetic dissection of SLE pathogenesis: adoptive transfer of Sle1 mediates the loss of tolerance by bone marrow-derived B cells. *J.Immunol.* **162**, 2415-2421.

Sobel, E.S., Yokoyama, W.M., Shevach, E.M., Eisenberg, R.A., and Cohen, P.L. (1993). Aberrant expression of the very early activation antigen on MRL/Mp-lpr/lpr lymphocytes. *J.Immunol.* **150**, 673-682.

Spinozzi, F., Agea, E., Bistoni, O., Travetti, A., Migliorati, G., Moraca, R., Nicoletti, I., Riccardi, C., Paoletti, F.P., and Vaccaro, R. (1995). T lymphocytes bearing the gamma delta T cell receptor are susceptible to steroid-induced programmed cell death. *Scand.J.Immunol.* **41**, 504-508.

Springer, T.A. (1990). Adhesion receptors of the immune system. *Nature* **346**, 425-434.

Spronk P.E., Horst G., Vab Der Gun B.T., Limburg P.C., and Kallenberg C.G. (1996). Anti-dsDNA production coincides with concurrent B and T cell activation during development of active disease in systemic lupus erythematosus (SLE). *Clin.Exp.Immunol.* **104**, 446-453.

Spronk, P.E., Gun, B.T., Limburg, P.C., and Kallenberg, C.G. (1993). B cell activation in clinically quiescent systemic lupus erythematosus (SLE) is related to immunoglobulin levels, but not to levels of anti- dsDNA, nor to concurrent T cell activation. *Clin.Exp.Immunol.* **93**, 39-44.

Spronk, P.E., Horst, G., Van Der Gun, B.T., Limburg, P.C., and Kallenberg, C.G. (1996). Anti-dsDNA production coincides with concurrent B and T cell activation during development of active disease in systemic lupus erythematosus (SLE). *Clin.Exp.Immunol.* **104**, 446-453.

Steinberg, A.D., Roths, J.B., Murphy, E.D., Steinberg, R.T., and Raveche, E.S. (1980). Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. *J.Immunol.* **125**, 871-873.

Strasser, A., Whittingham, S., Vaux, D.L., Bath, M.L., Adams, J.M., Cory, S., and Harris, A.W. (1991). Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc.Natl.Acad.Sci.* **88**, 8661-8665.

Strober, S., Cheng, L., Zeng, D., Palathumpat, R., Dejbakhsh-Jones, S., Huie, P., and Sibley, R. (1996). Double negative (CD4-CD8- alpha beta+) T cells which promote tolerance induction and regulate autoimmunity. *Immunol.Rev.* **149**, 217-230.

Su, C.C., Shau, W.Y., Wang, C.R., Chuang, C.Y., and Chen, C.Y. (1997). CD69 to CD3 ratio of peripheral blood mononuclear cells as a marker to monitor systemic lupus erythematosus disease activity. *Lupus.* **6**, 449-454.

Sumida, T., Maeda, T., Taniguchi, M., Nishioka, K., and Stohl, W.(1998). TCR AV24 gene expression in double negative T cells in systemic lupus erythematosus [letter]. *Lupus.* **7**, 565-568.

Sumida, T., Sakamoto, A., Murata, H., Makino, Y., Takahashi, H., Yoshida, S., Nishioka, K., Iwamoto, I., and Taniguchi, M. (1995). Selective reduction of T cells bearing invariant V alpha 24J alpha Q antigen receptor in patients with systemic sclerosis. *J.Exp.Med.* **182**, 1163-1168.

Suzushima, H., Asou, N., Nishimura, S., Nishikawa, K., Wang, J.X., Okubo, T., Naito, M., Hattori, T., and Takatsuki, K. (1993). Double-negative (CD4- CD8-) T cells from adult T-cell leukemia patients also have poor expression of the T-cell receptor alpha beta/CD3 complex. *Blood* **81**, 1032-1039.

Takahashi, S., Fossati, L., Iwamoto, M., Merino, R., Motta, R., Kobayakawa, T., and Izui, S. (1996). Imbalance Towards Th1 Predominance is Associated with Acceleration of Lupus-like Autoimmune Syndrome in MRL Mice. *J. Clin. Invest.* **97**, 1597-1604.

Takeno, M., Nagafuchi, H., Kaneko, S., Wakisaka, S., Oneda, K., Takeba, Y., Yamashita, N., Suzuki, N., Kaneoka, H., and Sakane, T. (1997). Autoreactive T cell clones from patients with systemic lupus erythematosus support polyclonal autoantibody production. *J.Immunol.* **158**, 3529-3538.

Takiguchi, M., Murakami, M., Nakagawa, I., Saito, I., Hashimoto, A., and Uede, T. (2000). CTLA4IgG gene delivery prevents autoantibody production and lupus nephritis in MRL/lpr mice. *Life Sci.*. **66**, 991-1001.

Tan, E.M. (1989). Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv.Immunol.* **44:93-151.**, 93-151.

Tan, E.M., Cohen, A.S., Fries, J.F., Masi, A.T., McShane, D.J., Rothfield, N.F., Schaller, J.G., Talal, N., and Winchester, R.J. (1982). The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* **25**, 1271-1277.

Tang, Q., Smith, J.A., Szot, G.L., Zhou, P., Alegre, M.L., Henriksen, K.J., Thompson, C.B., and Bluestone, J.A. (2003). CD28/B7 regulation of anti-CD3-mediated immunosuppression in vivo. *J.Immunol.* **170**, 1510-1516.

Taylor, P.R., Carugati, A., Fadok, V.A., Cook, H.T., Andrews, M., Carroll, M.C., Savill, J.S., Henson, P.M., Botto, M., and Walport, M.J. (2000). A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J.Exp.Med.* **192**, 359-366.

Testi, R., D'Ambrosio, D., De Maria, R., and Santoni, A.(1994). The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol.Today* **15**, 479-483.

Theofilopoulos, A.N., Kofler, R., Singer, P.A., and Dixon, F.J.(1989). Molecular genetics of murine lupus models. *Adv.Immunol.* **46**, 61-109.

Theofilopoulos, A.N., Koundouris, S., Kono, D.H., and Lawson, B.R. (2001). The role of IFN-gamma in systemic lupus erythematosus: a challenge to the Th1/Th2 paradigm in autoimmunity. *Arthritis Res.* **3**, 136-141.

Thomas, M.L.(1989). The leukocyte common antigen family. *Annu.Rev.Immunol.* **7**, 339-369.

Tivol, E.A., Borriello, F., Schweitzer, A.N., Lynch, W.P., Bluestone, J.A., and Sharpe, A.H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* **3**, 541-547.

Tokano, Y., Morimoto, S., Hishikawa, T., Murashima, A., Abe, M., Sekigawa, I., Takasaki, Y., Hashimoto, H., Okumura, K., Shirai, T., and Hirose, S. (1997). Subsets of activated T cells in patients with systemic lupus erythematosus: the relation to cell cycle. *Scand.J.Rheumatol.* **26**, 37-42.

Toribio, M.L., de la Hera, A., Regueiro, J.R., Marquez, C., Marcos, M.A., Bragado, R., Arnaiz-Villena, A., and Martinez, C. (1988). Alpha/beta heterodimeric T-cell receptor expression early in thymocyte differentiation. *J.Mol.Cell Immunol.* **3**, 347-362.

Tsokos G.C.(1996). Lymphocytes, Cytokines, Inflammation and Immune Trafficking. *Current Opinion in Rheumatology* **8**, 395-402.

Tsokos, G.C., Kovacs, B., Sfikakis, P.P., Theocharis, S., Vogelgesang, S., and Via, C.S. (1996). Defective antigen-presenting cell function in patients with systemic lupus erythematosus. *Arthritis Rheum.* **39**, 600-609.

Van Bruggen, M.C., Kramers, C., Walgreen, B., Elema, J.D., Kallenberg, C.G., van den Born, J., Smeenk, R.J., Assmann, K.J., Muller, S., Monestier, M., and Berden, J.H. (1997). Nucleosomes and histones are present in glomerular deposits in human lupus nephritis. *Nephrol.Dial.Transplant.* **12**, 57-66.

Van Eden, W., Van Der Zee, R., Van Kooten, P., Berlo, S.E., Cobelens, P.M., Kavelaars, A., Heijnen, C.J., Prakken, B., Roord, S., and Albani, S. (2002). Balancing the immune system: Th1 and Th2. *Ann.Rheum.Dis.* **61,** Suppl 2:ii25-8.

Van Ghelue, M., Moens, U., Bendiksen, S., and Rekvig, O.P. (2003). Autoimmunity to nucleosomes related to viral infection: a focus on hapten-carrier complex formation. *J. Autoimmun.* **20**, 171-182.

Via, C.S., Tsokos, G.C., Bermas, B., Clerici, M., and Shearer, G.M. (1993). T cell-antigen-presenting cell interactions in human systemic lupus erythematosus. Evidence for heterogeneous expression of multiple defects. *J.Immunol.* **151**, 3914-3922.

Vitali, C., Bencivelli, W., Isenberg, D.A., Smolen, J.S., Snaith, M.L., Sciuto, M., d'Ascanio, A., and Bombardieri, S. (1992). Disease activity in systemic lupus erythematosus: report of the Consensus Study Group of the European Workshop for Rheumatology Research. I. A descriptive analysis of 704 European lupus patients. European Consensus Study Group for Disease Activity in SLE. *Clin.Exp.Rheumatol.* **10**, 527-539.

von Boehmer, H., Kirberg, J., and Rocha, B.(1991). An unusual lineage of alpha/beta T cells that contains autoreactive cells. *J.Exp.Med.* **174**, 1001-1008.

Wakeland, E.K., Liu, K., Graham, R.R., and Behrens, T.W. (2001). Delineating the genetic basis of systemic lupus erythematosus. *Immunity.* **15**, 397-408.

Walton, A.J., Snaith, M.L., Locnisker, M. (1991). Dietary fish oil and the severity of symptoms in patients with systemic lupus erythematosus. *Ann. Rheum.Dis.* **50**, 463-466.

Wang, X., Huang, W., Mihara, M., Sinha, J., and Davidson, A. (2002). Mechanism of action of combined short-term CTLA4Ig and anti-CD40 ligand in murine systemic lupus erythematosus. *J.Immunol.* **168**, 2046-2053.

Ward, M.M., Marx, A.S., and Barry, N.N. (2000). Comparison of the validity and sensitivity to change of 5 activity indices in systemic lupus erythematosus. *J.Rheumatol.* **27**, 664-670.

Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4 [see comments]. *Science* **270**, 985-988.

Wells, A.D., Walsh, M.C., Bluestone, J.A., and Turka, L.A. (2001). Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *J.Clin.Invest.*. **108**, 895-903.

Wilson, S.B., Kent, S.C., Patton, K.T., Orban, T., Jackson, R.A., Exley, M., Porcelli, S., Schatz, D.A., Atkinson, M.A., Balk, S.P., Strominger, J.L., and Hafler, D.A. (1998). Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. *Nature* **391**, 177-181.

Wofsy, D. and Seaman, W.E. (1987). Reversal of advanced murine lupus in NZB/NZW F1 mice by treatment with monoclonal antibody to L3T4. *J.Immunol.* **138**, 3247-3253.

Wu, Z., Podack, E.R., McKenzie, J.M., Olsen, K.J., and Zakarija, M. (1994). Perforin expression by thyroid-infiltrating T cells in autoimmune thyroid disease. *Clin.Exp.Immunol.* **98**, 470-477.

Yang, B.C., Wang, Y.S., Lin, L.C., and Liu, M.F. (1997). Induction of apoptosis and cytokine gene expression in T-cell lines by sera of patients with systemic lupus erythematosus. *Scand.J.Immunol.* **45**, 96-102.

Yang, L., Du, T.B., Khan, Q., and Zhang, L. (1998). Mechanisms of long-term donor-specific allograft survival induced by pretransplant infusion of lymphocytes. *Blood* **91**, 324-330.

Yoshimoto, T., Bendelac, A., Hu-Li, J., and Paul, W.E. (1995). Defective IgE production by SJL mice is linked to the absence of CD4+, NK1.1+ T cells that promptly produce interleukin 4. *Proc.Natl.Acad.Sci.U.S.A.* **92**, 11931-11934.

Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J., and Paul, W.E. (1995). Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science* **270**, 1845-1847.

Young, K.J., Yang, L., Phillips, M.J., and Zhang, L. (2002). Donor-lymphocyte infusion induces transplantation tolerance by activating systemic and graft-infiltrating double-negative regulatory T cells. *Blood.* **100**, 3408-3414.

Young, K.J. and Zhang, L.(2002). The nature and mechanisms of DN regulatory T-cell mediated suppression. *Hum.Immunol.* **63**, 926-934.

Zhang, Z.X., Young, K., and Zhang, L. (2001). CD3+CD4-CD8- alphabeta-TCR+ T cell as immune regulatory cell. *J.Mol.Med.* **79**, 419-27.

Ziegler, S.F., Ramsdell, F., and Alderson, M.R. (1994). The activation antigen CD69. *Stem.Cells* **12** , 456-465.

Zimmerman, R., Radhakrishnan, J., Valeri, A., Appel, G. (2001). Advances in treatment of lupus nephritis. *Annu. Rev. Med.* **52**, 63-78.

Zouali, M. (2002). B cell diversity and longevity in systemic autoimmunity. *Mol.Immunol.* **38**, 895-901.

++

PUBLICATIONS.

A.Anand, GS.Dean, A.C.Quereshi, D.A. Isenberg and P.M. Lydyard. 2002.
Characterisation of $CD3^+$ $CD4^-$ $CD8^-$ (double negative) T cells in patients with systemic lupus erythematosus: Activation markers. *Lupus.* **11** 493-500.

G.S. Dean, **A.Anand**, A.Blofeld, D.A. Isenberg and P.M. Lydyard. 2002.
Characterisation of $CD3^+$ $CD4^-$ $CD8^-$ (double negative) T cells in patients with systemic lupus erythematosus: production of IL-4. *Lupus.* **11** 501-507.

.

Lupus (2002) 11, 493–500
www.lupus-journal.com

PAPER

Characterization of $CD3^+CD4^-CD8^-$ (double negative) T cells in patients with systemic lupus erythematosus: activation markers

A Anand[1], GS Dean[1,2], K Quereshi[1], DA Isenberg[2] and PM Lydyard[1]*
[1]Department of Immunology and Molecular Pathology, Royal Free and University College School of Medicine, London, UK; and [2]Centre for Rheumatology, Department of Medicine, Royal Free and University College School of Medicine, London, UK

Systemic lupus erythematosus (SLE) is characterized by B cell hyperactivity and the production of autoantibodies, some of which (antibodies to dsDNA) are thought to be pathogenic. T helper cells drive the production of autoantibodies and the aim of this study is to characterize phenotypically a subpopulation of T cells (the $CD3^+CD4^-CD8^-$, double negative (DN) T cells) previously identified as helping to enhance anti-DNA antibodies in patients with SLE. Data were obtained using FACS staining of DN T cells that had been purified from PBMCs by magnetic bead separation.

The percentage of TCR $\alpha\beta^+$ DN T cells was found to be significantly higher in patients with SLE as compared with controls ($P = 0.02$), although there was no significant increase in total percentage of DN T cells, which includes TCR $\gamma\delta^+$ cells. Activation markers HLA-DR and CD69, the costimulatory molecule CD28 and CTLA-4 were all expressed on the surface of a higher percentage of DN T cells in patients with SLE than in patients with rheumatoid arthritis (RA) or healthy controls (HC). More DN T cells from patients with SLE were of CD45RA phenotype than was found in controls, while CD45RO-expressing cells were reduced. In addition, DN T cells from patients with SLE expressed significantly higher levels of HLA-DR ($P = 0.006$), CD28 ($P = 0.05$), CTLA4 ($P = 0.03$) and CD45RA ($P = 0.05$) on the cell surface than those from the CD4/8 population. Correlation of expression of the markers measured with various parameters of disease activity and severity showed that high levels of HLA-DR expression correlated with high circulating serum C3 (> 0.9 IU/ml), indicating that an activated phenotype is consistent with severe disease. *Lupus* (2002) **11**, 493–500.

Key words: activation markers; double negative; SLE; T cells

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease characterized serologically by B cell hyperactivity and a panoply of autoantibodies against nuclear, cytoplasmic and cell surface antigens. Since these autoantibodies are mainly of the IgG1 subclass, it is likely that T lymphocytes are essential in providing help to the autoantibody producing B cells. Evidence for T cell involvement in disease pathogenesis is illustrated by the association of SLE with particular major histocompatability complex (MHC) class II alleles and affinity maturation of IgG autoantibody production.[1] It is thought that T helper (T_H) cells drive the production of pathogenic anti-DNA autoantibodies in SLE and it has been shown, *in vitro*, that some of these are $CD4^-$ $CD8^-$, i.e. double negative (DN) T cells,[2] which have been shown to express either the $\alpha\beta$ T cell receptor (TCR) or $\gamma\delta$ TCR.[3,4] However, conflicting results have been obtained about whether or not there is an increase in DN T cells in patients with SLE.[5–7]

To understand the contribution of DN T cells to disease pathogenesis, we have determined the presence of DN T cells in the blood of patients with SLE and further characterized them in terms of activation

*Correspondence: PM Lydyard, Department of Immunology and Molecular Pathology, Royal Free and University College School of Medicine, Windeyer Research Institute, 46 Cleveland St, London W1T 4JF, UK.
Email: p.lydyard@ucl.ac.uk
Received 7 January 2002; accepted 10 June 2002

1011.91/0961203302lu235oa

state. Our studies have shown that, although there was no significant increase in absolute circulating DN T cells, there was a greater proportion of the $\alpha\beta$ DN T cell population in SLE. Furthermore, these cells were more activated than control DN T cells as determined by a number of activation markers.

Materials and methods

Patients and controls

Twenty two patients with SLE (21 females and one male aged 17–67 years, mean 42 years) were studied with informed consent. Each met four or more of the revised criteria of the American College of Rheumatology for the classification of rheumatic disease.[1] Disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) computerized index.[8] The index is based upon the 'physician's intention to treat' principle and divides lupus activity into eight organs or systems which are scored from A (most active) to E (never previously active). A global score determined using A = 9, B = 3, C = 1, D = 0, E = 0 has been successfully compared with other methods of calculating global scores,[9] and in this study patients scoring > 6 were deemed active and ≤ 6 inactive. Levels of circulating anti-dsDNA antibodies and C3 were measured during routine patient assessment. Serum levels of anti-dsDNA antibodies in excess of 50 IU/ml (Shield Diagnostics, Dundee) and levels of C3 less than 0.9 IU/ml (by laser nephelometry) are regarded as abnormal. For statistical analysis in this study, antids-DNA antibody levels of 100 IU/ml or more were considered to be high. Seventeen patients with rheumatoid arthritis (RA: 15 female, three male, aged 35–82 years, mean 59 years) who fulfilled four or more of the ARA criteria for rheumatoid arthritis[10] and 18 healthy controls (HC: 17 females, one male aged 18–45 years, mean 31 years) were also studied.

Preparation of double negative T cells

Peripheral blood mononuclear cells (PBMCs) were separated from venous blood by Ficoll-Hypaque (Nycomed) gradients within 60 min of collection. The $CD4^-$ $CD8^-$ T cell population was enriched by negative selection. Briefly, PBMCs were incubated with monoclonal antibodies (Mabs) to CD4 (UCHT-8), CD8 (UCHT-4), the B cell marker CD19 (BU-12) and the monocyte marker CD14 (MY-23) for 30 min at 4°C under previously established optimum conditions. The washed cells were then incubated with sheep anti-mouse IgG Dynabeads (Dynal) for 30 min at 4°C and the non-$CD4^-$ $CD8^-$ T cells removed with a magnet. The remaining cells were tested for purity and were always >95% pure DN T cells.

Immunofluorescence staining and flow cytometry

Indirect three-colour staining prior to FACS analysis was carried out. Cells were labelled with the first antibody (30 min, 4°C), which was either HLA-DR (Dako-Patts Denmark), CD28 (gift from Dr Y Latchmann), CTLA-4 (gift from Dr BM Bröker), CD45RA or CD45RO (gifts from Professor BM Chain) followed by FITC conjugated anti-mouse $F(ab')_2$ (Dakopatts A/S Denmark), washed (PBS containing 1% BSA and 0.1% sodium azide) and any free anti-mouse Ig binding sites blocked with normal mouse serum. A direct staining method was used for measuring surface expression of CD69 (Pharmingen), and in every case the cells were also stained using directly conjugated CD3-PE or TCR$\alpha\beta$-PE, CD4-Cy and CD8-Cy (Pharmingen) in order to identify the double negative T cells. Samples were then fixed for 10 min in 2% paraformaldehyde before analysis or storage at 4°C in the dark. It is possible to distinguish $CD4^+$ and $CD8^+$ cells from the remaining PBMCs and from each other using antibodies conjugated with the same fluorochrome (Cychrome in this case) because the two different cell populations are of different density and each can be visualized as a clearly distinguishable separate population on the screen. All antibodies were used at optimal saturation concentrations. Background staining was determined using isotype matched non-conjugated monoclonal antibodies and FITC conjugated anti-mouse antibody for indirect staining and isotype matched FITC, PE or Cy conjugated monoclonal antibodies (DakoPatts) for direct staining. Cells were analysed on a FACScan flow cytometer (Becton Dickinson) using a lymphocyte gate based on forward and side light scatter characteristics. All analyses were performed using WinMDI version 2.8 software.

Statistical analysis

Data were analysed using the non-parametric Mann–Whitney test and Wilcoxon's test for paired samples. A two-tailed non-parametric (Spearman) test with 95% confidence limits was used to determine correlations.

Results

Quantitation of double negative T cells

DN T cells were identified using three-colour immunofluorescence staining with CD3-PE, CD4-Cychrome and CD8-Cychrome used to distinguish the DN CD3 positive cells from total PBMCs (DN T cells are

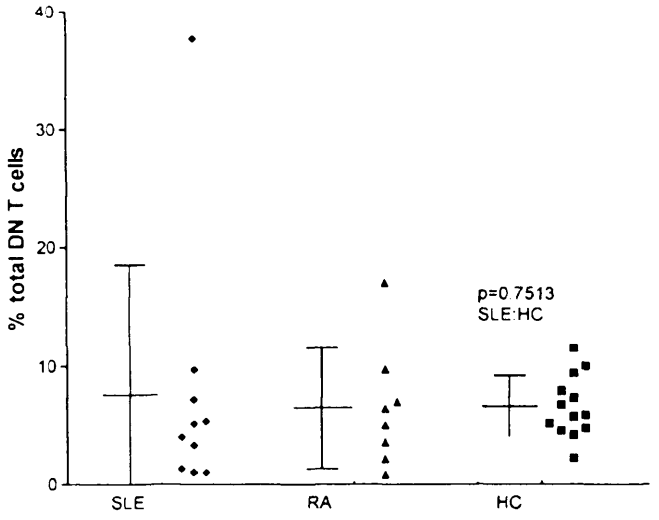


Figure 1 The percentages of blood DN T cells are similar in patients with SLE ($n = 10$), RA ($n = 8$) and HC ($n = 13$). Total blood PBMCs were stained with fluorescent antibodies to CD3, CD4 and CD8, for flow cytometric analysis, as described in Materials and methods.

$CD3^+$, $CD4^-$ and $CD8^-$) and the third colour being used to identify the other parameter being measured.

The percentages of DN T cells measured in the total PBMCs of SLE patients did not differ from control patients with RA or HC (Figure 1). However, on further analysis of the populations expressing either $\alpha\beta$ or $\gamma\delta$ T cell receptors, the percentages of $\alpha\beta$ TCR^+ DN T cells in SLE patients within the total DN population (59.81 ± 10.39) were significantly increased ($P = 0.04$) compared with RA (53.08 ± 21.91) and HC (48.24 ± 15.23; Figure 2).

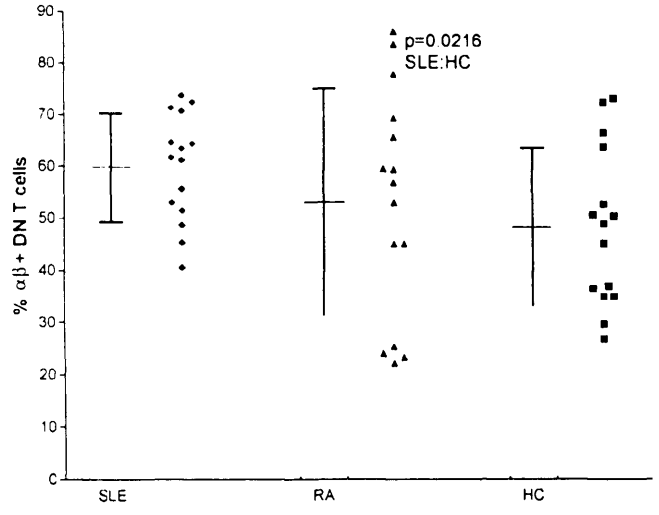


Figure 2 The percentages of $\alpha\beta^+$ DN T cells are increased in SLE patients ($n = 15$) compared with RA ($n = 15$) and HC($n = 15$). Blood PBMCs were triple stained for CD4/CD8, CD3 and $\alpha\beta$ as described in the Materials and methods. $P = 0.02$ for increase in $\alpha\beta+$ DN T compared with both RA and HC.

Detailed examination of the treatment regimes at the time of sampling showed that, while many of the patients were on steroid therapy, either alone or in combination with other drugs, eight were receiving no major drug therapy (Table 1).

Expression of surface markers on DN T cells in patients with SLE

A number of surface molecules were analysed on the DN T cells of patients with SLE, RA and HC (Figure 3).

HLA-DR. The percentages of $HLA\text{-}DR^+$ DN T cells were increased in patients with SLE compared with both RA patients ($P = 0.016$) and HC ($P = 0.01$; Figure 3a). In addition, there were significantly more $HLA\text{-}DR^+$ DN T cells than $CD4/8^+$ T cells in patients with SLE ($P = 0.006$; Figure 4a). However, there was neither any significant difference in percentages of $HLA\text{-}DR^+$ DN T cells nor of $CD4/8^+$ T cells in patients with active disease compared with inactive disease (Global Disease Activity Index). There was no correlation between Global Disease Activity Index and percentage of $HLA\text{-}DR^+$ $CD4/8^+$ T cells ($r = 0.21$, $P = 0.48$) or $HLA\text{-}DR^+$ DN T cells ($r = -0.08$, $P = 0.78$). With regard to anti-dsDNA antibody levels, there were no significant

Table 1 SLE patients and their treatments

Patient No.	*Date of Birth*	*Drug Treatment*
19	28.08.1928	No major drugs
5	30.04.1941	No major drugs
20	31.12.1942	No major drugs
8	08.05.1950	No major drugs
16	10.08.1950	No major drugs
10	26.09.1959	No major drugs
21	26.09.1959	No major drugs
1	18.07.1964	No major drugs
22	08.12.1973	No major drugs
18	07.08.1952	5 mg Prednisolone
9	09.02.1970	400 mg Hydroxychloroquine
13	16.08.1959	200 mg Plaquanil
6	02.09.1949	2 mg Prednisolone 200 mg Hydroxychloroquine
17	11.05.1952	2.5 mg Prednisolone 400 mg Plaquanil
4	20.06.1974	5 mg Prednisolone 400 mg Hydroxychloroquine
7	04.06.1960	8 mg Prednisolone 400 mg Hydroxychloroquine 750 mg quarterly Cyclophosphamide pulse
2	04.05.1962	10 mg Prednisolone 400 mg Hydroxychloroquine
12	26.01.1968	7.5 mg Prednisolone 25 mg Azathioprine
15	19.12.1969	7.5 mg Prednisolone 150 mg Azathioprine
11	21.12.1950	400 mg Hydroxycloroquine 125 mg monthly Deltastab pulse
3	28.08.1928	50 mg Azathioprine 125 mg monthly Deltastab pulse

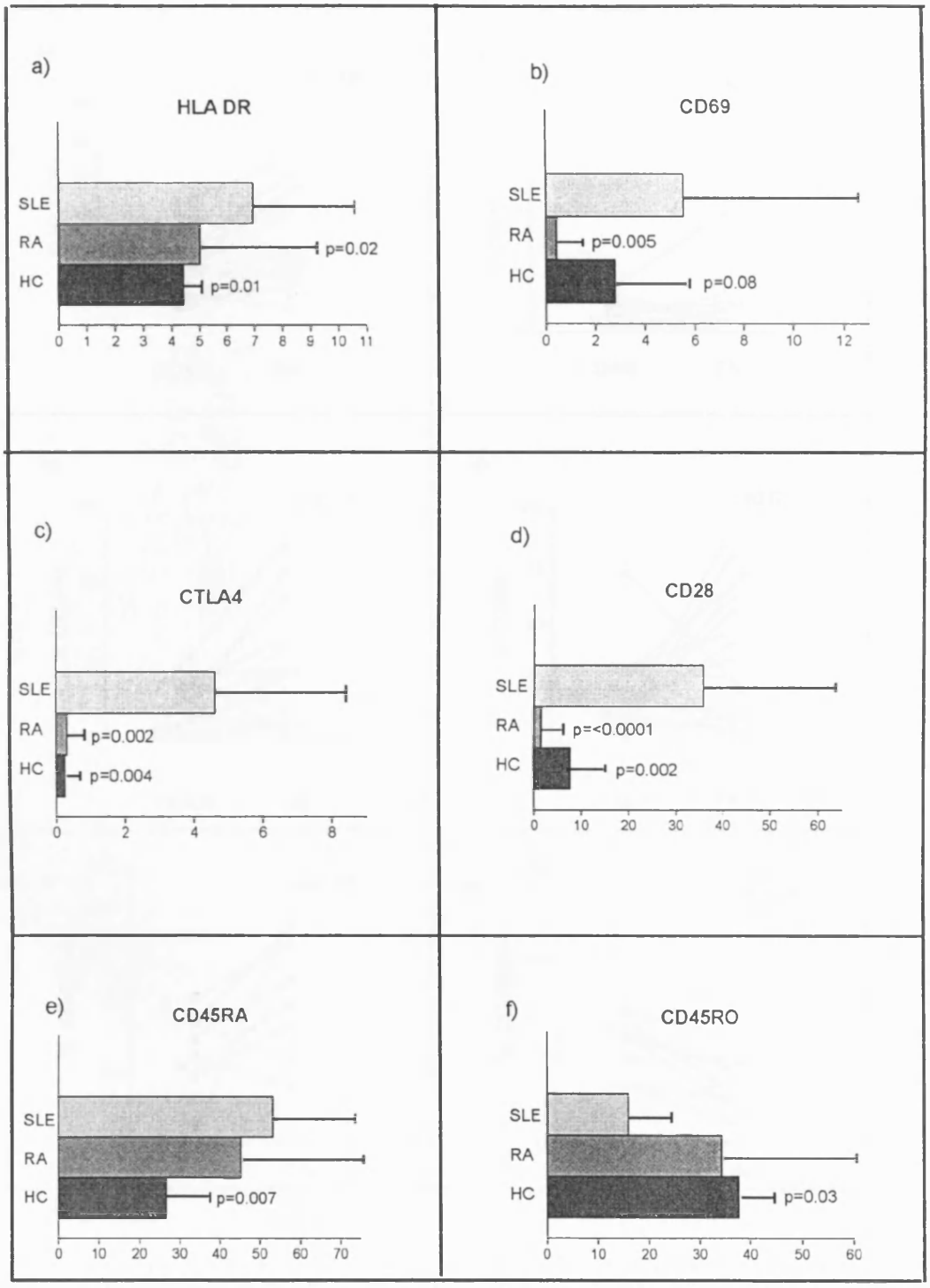


Figure 3 Expression of surface markers by DN T cells from patients with SLE, RA and HC. (a) HLA-DR expression is significantly increased in DN T cells from patients with SLE ($n = 17$) compared with either patients with RA ($n = 14$) or HC ($n = 13$). (b) CD69 expression is significantly increased in DN T cells from patients with SLE ($n = 11$) compared with patients with RA ($n = 11$). In HC ($n = 9$), expression of CD69 is lower but the difference is not significant. (c) Expression of CTLA4 is significantly increased in DN T cells from patients with SLE ($n = 14$) compared with either patients with RA ($n = 10$) or HC ($n = 8$). (d) CD28 expression is significantly increased in DN T cells from patients with SLE ($n = 17$) compared with either patients with RA ($n = 15$) or HC ($n = 12$). (e) Expression of CD45RA is significantly increased in patients with SLE ($n = 8$) compared with HC ($n = 6$). There was no difference in expression of CD45RA compared with patients with RA ($n = 12$). (f) Expression of CD45RO is significantly decreased in patients with SLE ($n = 7$) compared with HC ($n = 9$). There is no difference in expression of CD45RA compared with patients with RA ($n = 5$).

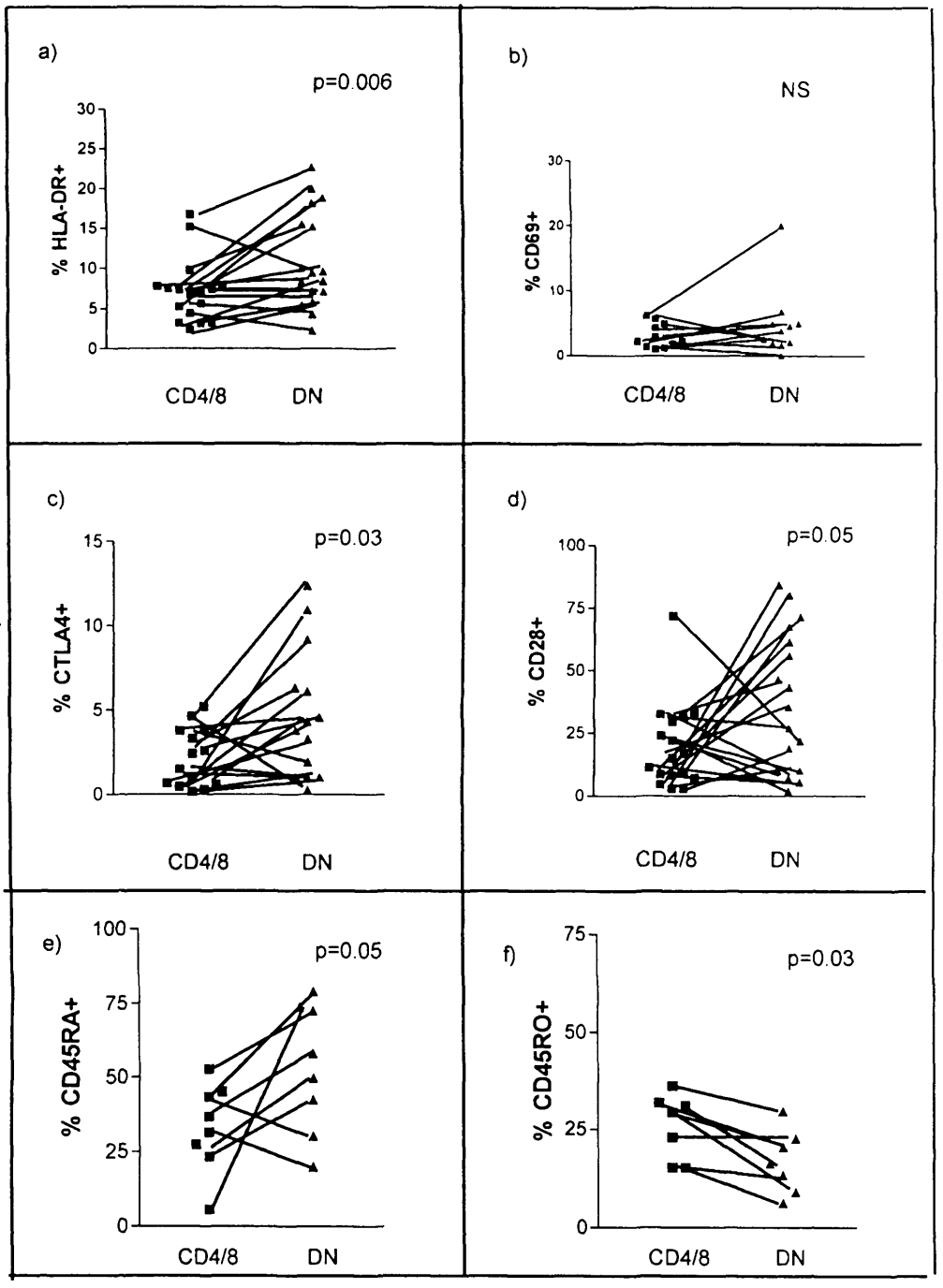


Figure 4 Expression of surface markers by CD4/8$^+$ T cells compared with DN T cells from patients with SLE. (a) Expression of HLA-DR is significantly higher in DN T cells than CD4/8$^+$ T cells ($n = 17$). (b) There is no significant difference in expression of CD69 between CD4/8$^+$ T cells and DN T cells ($n = 11$). (c) Expression of CTLA4 is significantly higher in DN T cells than CD4/8$^+$ T cells ($n = 14$). (d) CD28 expression is significantly higher in DN T cells than CD4/8$^+$ T cells ($n = 17$). (e) CD45RA expression is significantly higher in DN T cells than CD4/8$^+$ T cells ($n = 8$). (f) CD45RO expression is significantly lower in DN T cells than CD4/8$^+$ T cells ($n = 7$).

differences in percentages of HLA-DR$^+$ CD4/8$^+$ T cells or HLA-DR$^+$ DN T cells between patients with high *vs* low levels and no correlation between levels of anti-dsDNA antibody and percentage of HLA-DR$^+$ CD4/8$^+$ T cells ($r = 0.35$, $P = 0.25$) or HLA-DR$^+$ DN T cells ($r = -0.38$, $P = 0.19$). However, when high and low levels of circulating C3 were related to HLA-DR expression, HLA-DR expression

was significantly higher in DN T cells from patients with high C3 ($P = 0.03$). There was a positive correlation between C3 level and HLA-DR expression in both CD4/8$^+$ T cells and DN T cells ($r = 0.57$, $P = 0.04$ and $r = 0.58$, $P = 0.04$, respectively).

CD69. Although patients with SLE showed an increased percentage of DN T cells expressing CD69 compared to HC, this was not significant ($P = 0.08$; Figure 2b). However, there was a significant increase in CD69$^+$ DN T cells in SLE compared with RA patients ($P = 0.005$). In addition, in the case of RA there was a significantly lower percentage of CD69$^+$ DN T cells compared with the CD69$^+$ CD4/8$^+$ T cell population ($P = 0.004$, data not shown). In patients with SLE, the percentages of DN CD69$^+$ cells were not significantly different from the percentages of CD4/8$^+$ CD69$^+$ T cells (Figure 4b). There was no significant difference in CD69$^+$ DN T cells or CD69$^+$ CD4/8$^+$ T cells in those patients with active disease compared with inactive disease. In addition, there was no correlation between either CD69$^+$ DN or CD69$^+$ CD4/8$^+$ expression and Global Disease Activity Index ($r = 0.21$, $P = 0.54$ and $r = -0.55 - 0.52$, $P = 0.95$, respectively). No significant differences in percentages of CD69$^+$ DN or CD69$^+$ CD4/8$^+$ were seen between high and low levels of serum anti-dsDNA and no correlation was found between percentage of CD69$^+$ CD4/8$^+$ T cells ($r = 0.05$, $P = 0.87$) or CD69$^+$ DN T cells ($r = 0.33$, $P = 0.31$) and level of anti-dsDNA antibody. No significant differences were seen between low and high levels of serum C3 and percentage of CD69$^+$ DN or CD69$^+$ CD4/8$^+$ and no correlation between percentage of either CD69$^+$ DN ($r = 0.49$, $P = 0.12$) or CD69$^+$ CD4/8$^+$ ($r = -0.38$, $P = 0.25$) and level of anti-dsDNA antibody.

CTLA4. Although few CTLA4$^+$ cells were detected (Figure 3c), there were significantly more in the DN T cell population of the patients with SLE compared with both the RA patients ($P = 0.002$) and HC ($P = 0.004$). Numbers of CTLA4$^+$ CD4/8$^+$ T cells also appeared to be increased compared with control groups, but the increase was not statistically significant. The percentages of CTLA4$^+$ DN T cells were significantly higher than the CTLA4$^+$ CD4/8$^+$ cells in both the SLE patients ($P = 0.03$; Figure 4c) and HC ($P = 0.001$; data not shown).

CD28. There were more DN T cells expressing the costimulatory molecule CD28 in SLE patients than in either RA ($P = < 0.0001$) or HC ($P = 0.002$; Figure 3d). Higher levels of CD28$^+$ DN T cells were seen compared with CD28$^+$CD4/8$^+$ T cells populations in SLE ($P = 0.05$; Figure 4d).

CD45RA/RO. DN T cells from patients with SLE showed significantly increased percentages of CD45RA$^+$ compared with HC ($P = 0.007$) but not with RA controls (Figure 3e). Conversely, SLE patients had significantly lower percentages of CD45RO$^+$ T cells in comparison with HC ($P = 0.03$), but not with RA controls (Figure 3f). In patients with SLE, the DN population was significantly increased in CD45RA compared with the CD4/8$^+$ population ($P = 0.048$; Figure 4e), while it was significantly decreased in CD45RO ($P = 0.03$; Figure 4f).

We had insufficient data to determine accurately whether there was any correlation between disease activity, anti-dsDNA or C3 and the levels of expression of CD28, CTLA4, CD45RA or CD45RO.

Discussion

In this study we show evidence for differences in the DN T cell population in patients with SLE compared with this population in RA patients and HC. Double negative T cells have been shown to express either the $\alpha\beta$ T cell receptor (TCR) or $\gamma\delta$TCR.[3,11] Although there was no increase in the levels of the total DN T cell populations in SLE, we found that TCR $\alpha\beta^+$ DN T cells were present in higher levels in patients with SLE compared with HC ($P = 0.02$). There was no significant difference in numbers of TCR $\alpha\beta^+$ DN T cells in patients with RA compared with SLE. Other investigators have shown an increase in DN T cells in various autoimmune diseases including in patients with SLE.[6,12] It is unlikely that the observed increase was a phenomenon induced by therapy because, although many of the patients were receiving steroids, either alone or in combination with other drugs, eight patients were not receiving any major drugs. The expression of activation markers by the DN T cells in patients with SLE could indicate that they are functionally important in the disease. The percentages of cells expressing CD69 in the DN T cell population of patients with SLE was higher compared with autoimmune (RA) and healthy controls ($P = 0.005$). CD69 is one of the earliest cell activation antigens that is expressed by activated lymphocytes and monocytes[13] and is involved in signal transduction, cell proliferation and cytokine secretion.[14] To our knowledge, our studies are the first to investigate CD69 expression by DN T cells in SLE. Other investigators have, however, shown that numbers of CD69$^+$ cells are marginally increased in freshly isolated CD4$^+$ or CD8$^+$ T cells from patients with SLE, especially in

the $CD8^+$ T cell compartment.[15–17] It has also been reported that the CD69/CD3 ratio, which detects T cell activation, is correlated with disease activity in patients with SLE.[18] In our studies, the differences between the DN T cell populations were much more significant than the differences seen comparing the $CD4/CD8^+$ populations ($P = 0.12$) suggesting that the level of expression of CD69, although increased on the overall T cell populations, was highest for the DN T cells in SLE patients.

HLA-DR is mainly expressed by T cells 24–48 h after activation. Increased percentages of DN T cells expressing HLA-DR in patients with SLE compared with the two control groups ($P = 0.02$ RA and $P = 0.01$ HC), like the expression of CD69, indicates that there is a on-ongoing activation of these cells in the patients. Although we found no significant differences in HLA-DR expression between patients with SLE and the control groups in the $CD4/8^+$ population, others have reported a higher proportion of $HLA\text{-}DR^+$ T cells in both the $CD4^+$ and $CD8^+$ T cells in patients with SLE.[17,18] It is unclear why we did not find differences in expression of the overall $CD4/CD8^+$ cells and unlikely that our findings are due to the disease activity of our patient cohort as there does not appear to be any correlation between Global Disease Activity Index and HLA-DR expression. However, we did find a positive correlation between C3 levels and HLA-DR expression in both $CD4/8^+$ T cells and DN T cells. Higher levels of circulating C3 are indicative of less active disease and this finding might indicate that highly activated DN T cells could have a protective effect.

CD28 has been shown to be present on the majority of $CD4^+$ T cells with fewer $CD8^+$ T cells expressing this molecule.[19] It has been reported that patients with SLE have a preferential decrease of $CD28^+$ T cells, especially in the $CD8^+$ T cell compartment.[20,21] We analysed the $CD4^+$ and $CD8^+$ cells together and found there to be significantly more $CD28^+$ cells in the DN compartment than in the $CD4/8^+$ population ($P = 0.045$). We also found that more DN T cells expressed CD28 in patients with SLE compared to RA or HC. The significance of this is currently unclear but engagement of CD28 on T cells by its counter-receptors B7-1 (CD80) and B7-2 (CD86) on accessory cells is pivotal for T cell activation and in the absence of this costimulation T cells are anergized.[19] Interestingly a defective CD28/B7 costimulatory pathway has also been described in SLE T cells, which could contribute to the disease pathogenesis.[20–22] Following activation via CD28, $CD4^+$ and $CD8^+$, T cells express CTLA-4 which has a higher affinity for the same ligands as CD28 (CD80/CD86). This is thought to deliver a negative signal to the T cells.[23–25] Our studies on CTLA-4 expression by $CD4/CD8^+$ T cells in SLE patients have shown increased percentages of $CTLA\text{-}4^+$ T cells compared with normal controls, consistent with that reported by others.[26] In addition, we showed a low but significantly increased percentage of DN T cells expressing CTLA-4 in our patients with SLE. This concomitant increase in the number of cells expressing both CD28 and CTLA-4 confirms the highly activated status of some DN T cells in patients with SLE. CTLA-4 is often detected intracellularly. However, in this study we did not stain for intracellular CTLA-4 routinely because in three patients studied we found no significant differences in the results for surface or intracellular staining (data not shown). Expression of CD45RO usually means that the cells are 'antigen experienced' and those that are CD45RA are 'antigen naive'. We found that a significantly higher percentage of DN T cells but not $CD4/CD8^+$ T cells expressed CD45RA in patients with SLE compared with HC ($P = 0.007$). The patients also showed a significantly higher proportion of $CD45RA^+$ cells to $CD45RO^+$ cells for $CD4/CD8^+$ T cells ($P = 0.05$) and DN T cells ($P = 0.05$). One explanation for this could be the recruitment of naive/resting DN T cells to the ongoing immune reaction in patients with SLE rather than the reactivation of existing memory clones. This finding is in agreement with the results of studies following the course of disease from the onset of symptoms in patients with SLE.[29] We conclude that the DN T cell population is heterogeneous, in that, while some DN T cells in SLE show increased expression of a number of activation markers, suggesting some *in vivo* activation, others do not. It is possible that the activated cells may provide help for antibody and autoantibody production, although this theory was not tested in the study. Interestingly, studies of autoreactive T cell lines or clones capable of inducing anti-dsDNA production *in vitro* which had been derived from patients with SLE showed these cells to be either $CD4^-CD8^-TCR\alpha\beta^+$ or $CD4^+TCR\alpha\beta^+$ (T helper cells).[3,4] These cells could therefore provide help for autoantibody production in addition to the conventional $CD4^+$ helper T cells. This is consistent with the finding that DN T cells are enriched in IL4 producing cells in patients with SLE.[28–30]

What is activating some DN T cells in patients with SLE is unclear. Interestingly, recent studies have shown that DN T cells from SLE recognize CD1c expressed on B cells and can help the B cells to develop into IgG antibody producing cells.[31] CD1c is capable of presenting unconventional antigens such as a variety of foreign lipids and glycolipids.[32,33] This has led to speculation that the DN T cells can contribute to production of autoantibodies by recognizing

nucleosomes and could contribute to the development of anticardiolipin antibodies. The recent finding that NK-T cells within the DN T cell population express a TCR of $V\alpha24J\alpha Q^{3-4}$ has indicated that these cells may regulate progress of the disease. Further studies will analyse the characteristics of the $V\alpha24J\alpha Q$ TCR$^+$ DN T cells in controls and determine what role they have in patients with SLE.

References

1 Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271–1277.

2 Matsumoto M, Yasukawa M, Inatsuki A, Kobayashi Y. Human double-negative ($CD4^-CD8^-$) T cells bearing alpha beta T cell receptor possess both helper and cytotoxic activities. *Clin Exp Immunol* 1991; **85**: 525–530.

3 Rajagopalan S, Zordan T, Tsokos GC, Datta SK. Pathogenic anti-DNA autoantibody-inducing T helper cell lines from patients with active lupus nephritis: isolation of $CD4^-8^-$ T helper cell lines that express the gamma delta T-cell antigen receptor. *Proc Natl Acad Sci USA* 1990; **87**: 7020–7024.

4 Shivakumar S, Tsokos GC, Datta SK. T cell receptor alpha/beta expressing double-negative ($CD4^-/CD8^-$) and CD4+ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. *J Immunol* 1989; **143**: 103–112.

5 Brooks EG, Balk SP, Aupeix K, Colonna M, Strominger JL, Groh-Spies V. Human T-cell receptor (TCR) alpha/beta + $CD4^-CD8^-$ T cells express oligoclonal TCRs, share junctional motifs across TCR V beta-gene families, and phenotypically resemble memory T cells. *Proc Natl Acad Sci USA* 1993; **90**: 11787–11791.

6 Simon HU, Yousefi S, Dommann-Scherrer CC *et al.* Expansion of cytokine-producing $CD4^-CD8^-$ T cells associated with abnormal Fas expression and hypereosinophilia. [See comments.] *J Exp Med* 1996; **183**: 1071–1082.

7 Kusunoki Y, Hirai Y, Kyoizumi S, Akiyama M. Evidence for in vivo clonal proliferation of unique population of blood $CD4^-/CD8^-$ T cells bearing T-cell receptor alpha and beta chains in two normal men. *Blood* 1992; **79**: 2965–2972.

8 Hay EM, Bacon PA, Gordon C *et al.* The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. *Q J Med* 1993; **86**: 447–458.

9 Gladman DD, Goldsmith CH, Urowitz MB *et al.* Sensitivity to change of 3 systemic lupus erythematosus disease activity indices: international validation. *J Rheumatol* 1994; **21**: 1468–1471.

10 Arnett FC, Edworthy SM, Bloch DA *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; **31**: 315–324.

11 Shivakumar S, Tsokos GC, Datta SK. T cell receptor alpha/beta expressing double-negative ($CD4^-/CD8^-$) and $CD4^+$ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. *J Immunol* 1989; **143**: 103–112.

12 Kusunoki Y, Hirai Y, Kyoizumi S, Akiyama M. Evidence for in vivo clonal proliferation of unique population of blood $CD4^-/CD8^-$ T cells bearing T-cell receptor alpha and beta chains in two normal men. *Blood* 1992; **79**: 2965–2972.

13 Testi R, D'Ambrosio D, De Maria R, Santoni A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol Today* 1994; **15**: 479–483.

14 Cebrian M, Yague E, Rincon M, Lopez-Botet M, de Landazuri MO, Sanchez-Madrid F. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. *J Exp Med* 1988; **168**: 1621–1637.

15 Crispin JC, Martinez A, de Pablo P, Velasquillo C, Alcocer-Varela J. Participation of the CD69 antigen in the T-cell activation process of patients with systemic lupus erythematosus. *Scand J Immunol* 1998; **48**: 196–200.

16 Sakata K, Sakata A, Vela-Roch N *et al.* Fas (CD95)-transduced signal preferentially stimulates lupus peripheral T lymphocytes. *Eur J Immunol* 1998; **28**: 2648–2660.

17 Portales-Perez D, Gonzalez-Amaro R, Abud-Mendoza C, Sanchez-Armass S. Abnormalities in CD69 expression, cytosolic pH and $Ca2^+$ during activation of lymphocytes from patients with systemic lupus erythematosus. *Lupus* 1997; **6**: 48–56.

18 Su CC, Shau WY, Wang CR, Chuang CY, Chen CY. CD69 to CD3 ratio of peripheral blood mononuclear cells as a marker to monitor systemic lupus erythematosus disease activity. *Lupus* 1997; **6**: 449–454.

19 Gause WC, Halverston MJ, Lu P *et al.* The function of costimulatory molecules and the development of IL-4 producing T cells. *Immunology Today* 1997; **18**: 115–119.

20 Horwitz DA, Tang FL, Stimmler MM, Oki A, Gray JD. Decreased T cell response to anti-CD2 in systemic lupus erythematosus and reversal by anti-CD28: evidence for impaired T cell-accessory cell interaction. *Arthritis Rheum* 1997; **40**: 822–833.

21 Kaneko H, Saito K, Hashimoto H, Yagita H, Okumura K, Azuma M. Preferential elimination of $CD28^+$ T cells in systemic lupus erythematosus (SLE) and the relation with activation-induced apoptosis. *Clin Exp Immunol* 1996; **106**: 218–229.

22 Garcia-Cozar FJ, Molina IJ, Cuadrado MJ, Marubayashi M, Pena J, Santamaria M. Defective B7 expression on antigen-presenting cells underlying T cell activation abnormalities in systemic lupus erythematosus (SLE) patients. *Clin Exp Immunol* 1996; **104**: 72–79.

23 Castan J, Tenner-Racz K, Racz P, Fleischer B, Broker BM. Accumulation of CTLA-4 expressing T lymphocytes in the germinal centres of human lymphoid tissues. *Immunology* 1997; **90**: 265–271.

24 Waterhouse P, Penninger JM, Timms E *et al.* Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. [See comments.] *Science* 1995; **270**: 985–988.

25 Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 1995; **3**: 541–547.

26 Liu MF, Liu HS, Wang CR, Lei HY. Expression of CTLA-4 molecule in peripheral blood T lymphocytes from patients with systemic lupus erythematosus. *J Clin Immunol* 1998; **18**: 392–398.

27 Gordon C, Matthews N, Schlesinger BC *et al.* Active systemic lupus erythematosus is associated with the recruitment of naive/resting T cells. *Br J Rheumatol* 1996; **35**: 226–230.

28 Akahoshi M, Nakashima H, Tanaka Y *et al.* Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. *Arthritis Rheum* 1999; **42**: 1644–1648.

29 Funauchi M, Ikoma S, Enomoto H, Horiuchi A. Decreased Th1-like and increased Th2-like cells in systemic lupus erythematosus. *Scand J Rheumatol* 1998; **27**: 219–224.

30 Funauchi M, Yu H, Sugiyama M *et al.* Increased interleukin-4 production by NK T cells in systemic lupus erythematosus. *Clin Immunol* 1999; **92**: 197–202.

31 Sieling PA, Porcelli SA, Duong BT *et al.* Human double-negative T cells in systemic lupus erythematosus provide help for IgG and are restricted by CD1c. *J Immunol* 2000; **165**: 5338–5344.

32 Porcelli SA. The CD1 family: a third lineage of antigen-presenting molecules. *Adv Immunol* 1995; **59**: 1–98.

33 Porcelli SA, Modlin RL. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *A Rev Immunol* 1999; **17**: 297–329.

34 Oishi Y, Sumida T, Sakamoto A *et al.* Selective reduction and recovery of invariant Valpha24JalphaQ T cell receptor T cells in correlation with disease activity in patients with systemic lupus erythematosus. *J Rheumatol* 2001; **28**: 275–283.

Lupus (2002) 11, 501–507
www.lupus-journal.com

PAPER

Characterization of $CD3^+CD4^-CD8^-$ (double negative) T cells in patients with systemic lupus erythematosus: production of IL-4

GS Dean[1,2], A Anand[1], A Blofeld[1], DA Isenberg[2] and PM Lydyard[1]*
[1]Department of Immunology and Molecular Pathology, Royal Free and University College School of Medicine, London, UK; and
[2]Division of Rheumatology, Department of Medicine, Royal Free and University College School of Medicine, London, UK

Systemic lupus erythematosus (SLE) is a chronic autoimmune rheumatic disease that may affect every organ or system in the body. We have shown previously that the TCR $\alpha\beta^+$ subpopulation of $CD3^+CD4^-CD8^-$, DN T cells is expanded in patients with SLE and that double negative T cells express increased levels of activation markers compared both with healthy people and with patients with rheumatoid arthritis, (RA) as autoimmune controls. The aim of this study was to characterize these cells in terms of their ability to produce IL4, a Th2 cytokine, both spontaneously and after mitogen stimulation. It was found that a higher percentage of TCR $\alpha\beta^+$ double negative T cells from patients with SLE contained IL4 constitutively than did the same population of cells from healthy people or from those with RA. After mitogen stimulation, there was no significant difference in the amount of IL4 produced by each of the three groups. Further study of patients producing high levels of IL4 (about one third of the patients) indicated that they had a lower percentage of $\alpha\beta^+$ T cells in the double negative compartment than did patients with fewer IL4 containing cells. *Lupus* (2002) **11**, 501–507.

Key words: double negative; IL4; SLE; T cells

Introduction

SLE is a chronic autoimmune rheumatic disease that is characterized by B cell hyperactivity and the production of a spectrum of autoantibodies. The most important of these autoantibodies (because some are pathogenic), are the IgG antibodies specific for double-stranded DNA (anti-dsDNA). Several groups have shown a link between these antibodies and kidney disease, one of the most serious clinical manifestations of SLE.[1]

Double negative T cells (DN T) are thought to be a separate lineage of T cells that develop extrathymically in man.[2] We have previously characterized these cells in terms of their expression of activation markers and CD45 phenotype[3] and have shown that the $\alpha\beta^+$ DN T cells are increased as a percentage of total T cells in patients with SLE. Our work indicated that $\alpha\beta^+$ DN T cells in patients with SLE expressed higher levels of early (CD69) and late (HLA-DR) activation markers than controls. This subpopulation of T cells also appeared to contain a higher proportion of antigen naive cells ($CD45RA^+$) in SLE than in controls. $\alpha\beta^+$ DN T cells appear to have a function in the development of autoimmune disease and it is thought that their apparent autoreactive nature may be as a result of avoidance of the process of thymic selection that would delete autoreactive T cells. TCR$\alpha\beta^+$ DN T cells have been implicated in the pathogenesis of SLE and other autoimmune diseases in man as well as in various autoimmune conditions in mouse models.[4–6]

The aim of this study was to determine the percentage $\alpha\beta^+$DN T cells from patients with SLE and controls that contained intracellular IL4 and to

*Correspondence: PM Lydyard, Department of Immunology and Molecular Pathology, Royal Free and University College School of Medicine, Windeyer Research Institute, 46 Cleveland St, London W1T 4JF, UK.
E-mail: p.lydyard@ucl.ac.uk
Received 7 January 2002; accepted 10 June 2002

1011.91/0961203302lu234oa

compare these levels of cytokine production with those from the $CD4^+$ and $CD8^+$ $\alpha\beta^+$ T cell subpopulations.

Materials and methods

Patients and controls

Fifty patients with SLE (49 female and one male, aged from 17 to 66 years, mean age 37.2 years) were studied with informed consent. Each met four or more of the revised criteria of the American College of Rheumatology (ACR) for the classification of rheumatic disease.[7] Disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) computerized index.[8] The index is based on the 'physician's intention to treat' principle and divides lupus activity into eight organs or systems which are scored from A (most active) to E (never previously active). A BILAG global score (GS) determined using A = 9, B = 3, C = 9, D = 0, E = 0 has been successfully compared with other methods ofcalculating global scores,[9] and in this study patients were divided arbitrarily into two groups, active ($GS > 6$) and inactive ($GS \leq 6$). Levels of circulating anti-dsDNA antibodies and C3 were measured during routine patient assessment. Serum levels of anti dsDNA antibody levels in excess of 50 IU/ml (Shield Diagnostics, Dundee) and levels of C3 less than 0.9 IU/ml (by laser nephelometry) were regarded as abnormal. For statistical analysis in this study, anti dsDNA antibody levels of 100 IU/ml were considered to be high. Fifteen patients with RA (eight male and seven female, aged from 26 to 77 years, mean age 50.7 years) who fulfilled four or more of the ACR criteria for rheumatoid arthritis[10] and 16 healthy controls (HC, 13 female and three male, aged 21–57 years, mean age 36.1 years) were also studied.

Preparation of PBMCs

Blood was taken by venepuncture and PBMCs isolated using gradient centrifugation (Lymphoprep, Nycomed). Cells were washed three times and stained for expression of TCR$\alpha\beta$, CD4, CD8 and intracellular IL4. Peripheral blood mononuclear cells from a statistical sample of patients were incubated overnight at 37°C, 4.5% CO_2 in the presence of 10 μg/ml PHA (phytohaemagglutinin, Sigma). Monensin (0.05 M, Sigma) was added for the final 6 h to facilitate accumulation of cytokine in the Golgi[11] and the cells were stained for surface markers and intracellular IL4 as before. All of the antibodies used were directly conjugated and were obtained from Pharmingen or eBioscience (TCR$\alpha\beta$ (FITC), CD4 (Cy-5), CD8 (CY-5)) or R&D Systems (IL4 (PE)). Cells taken from some patients and controls were also stained for the activation markers, CD69 and HLA-DR (Pharmingen (PE)). $\alpha\beta^+$ DN T cells were identified by staining with TCR$\alpha\beta$ PE CD4 and CD8 CY-5 (two colours) and the $\alpha\beta^+CD4^-CD8^-$ population gated to determine expression of IL4 or surface markers using PE-conjugated antibodies.

Immunofluorescence staining and flow cytometry

Surface staining was carried out as previously described.[3] Cells were then fixed and permeabilized using Caltag Fix and Perm (Caltag) in accordance with the manufacturer's instructions prior to intracellular staining for IL4. After staining, cells were resuspended in 2% paraformaldehyde prior to immediate analysis or storage at 4°C in the dark. All antibodies were used at predetermined optimal saturating concentrations. Background staining was determined using isotype and conjugate-matched irrelevant antibodies obtained from the appropriate manufacturer. Cells were analysed on a FACScan flow cytometer (Becton Dickinson) using a lymphocyte live gate based on forward and side light scatter and counting a minimum of $10^4 - 10^5$ events. All analyses were performed using WinMDI version 2.8 software.

Statistical analysis

Data were analysed using the non-parametric Mann–Whitney test and the Wilcoxon non-parametric paired test. Correlation coefficients were calculated using a two-tailed non-parametric (Spearman) test with 95% confidence limits. Chi-squared was calculated using Fischer's exact test.

Results

Production of IL4 by $\alpha\beta^+$ DN, $CD4^+$ and $CD8^+$ T cells

Constitutive levels of IL4. $\alpha\beta^+$ DN T cells isolated from patients with SLE had significantly higher percentages of IL4-containing cells than those from patients with RA ($P = 0.003$, Figure. 1). The difference between patients with SLE and HC was not significant ($P = 0.066$). However, 12 of the patients with SLE were shown to have a percentage of $\alpha\beta^+$ DN T cells containing IL4 of more than twice the standard error of the mean of HC (Figure 1). The number of patients with SLE who showed high percentages of IL4 containing cells was significantly different from HC by Fischer's exact test ($P = 0.025$). In addition, it was observed that the percentage of

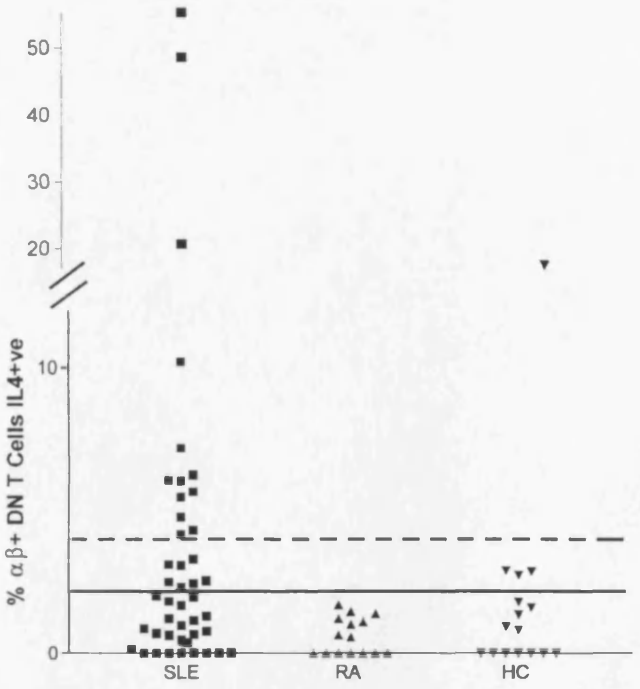


Figure 1 Percentage of IL4$^+$ $\alpha\beta^+$ DN T cells in the blood of patients with SLE ($n = 50$), RA ($n = 15$) and HC ($n = 16$). PBMCs were stained for CD4/CD8, $\alpha\beta$ TCR and IL4 as described in the Materials and methods. The dotted line represents +2 s.e.m. of patients with SLE.

IL4-positive cells was significantly higher in the $\alpha\beta^+$ DN T cell subset than in either the CD4$^+$ or the CD8$^+$ T cell subsets of patients with SLE both in whole population analyses ($P < 0.0001$ in each case) and in the 12 patients with IL4-positive cells greater than twice the standard error of the mean of HC ($P = 0.006$ for CD8$^+$ and $P = 0.019$ for CD4$^+$; data not shown). It was difficult to determine whether increased numbers of IL4$^+$ $\alpha\beta^+$ DN T cells in patients compared with controls also showed these individual cells to be producing more IL4 owing to variations in binding of isotype controls and anti-IL4 antibody from patient to patient.

IL4 production after PHA stimulation. When cells from these experimental groups were stimulated by overnight incubation with PHA, the differences in frequency of IL4-positive cells between the groups were no longer observable (Figure 2). Analysis of paired data from constitutive and PHA-stimulated cells showed a significant increase in percentage of IL4-positive $\alpha\beta^+$ DN T cells on stimulation for SLE ($P = 0.03$), RA ($P = 0.006$) and HC ($P = 0.004$). There was no significant difference in percentage of IL4-positive cells either between SLE and RA ($P = 0.8$) or between SLE and HC ($P = 0.9$). It was noted that, while most individuals tested showed an increase in IL4-positive cells following

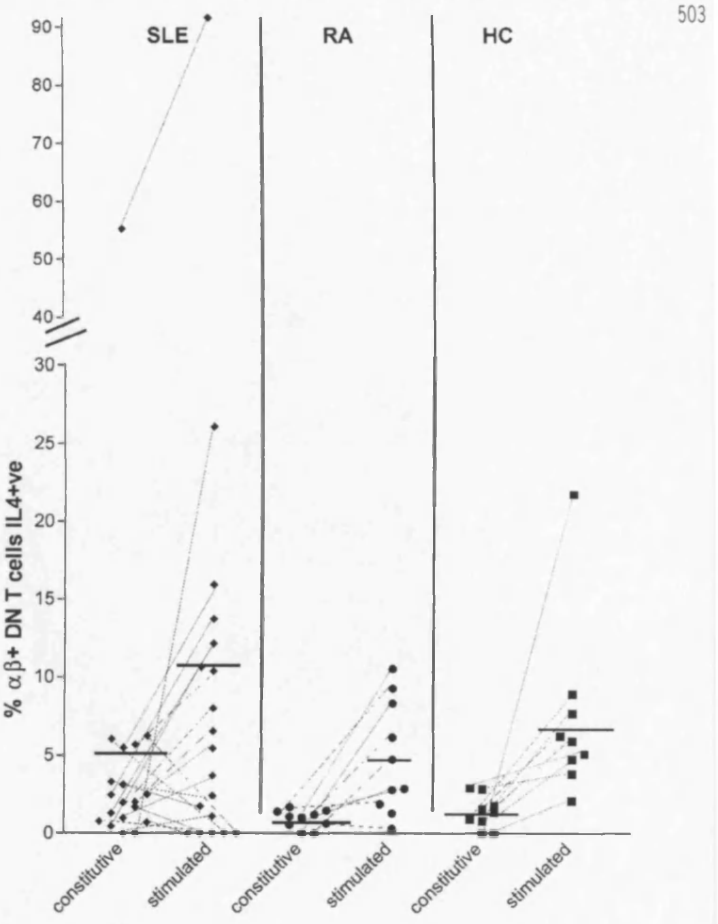


Figure 2 Effect of PHA on expression of IL4 by $\alpha\beta^+$ DN T cells with SLE, RA and HC. PBMCs were cultured with PHA for 24 h as described in the Materials and methods (stimulated) and stained as described in Figure 1 (constitutive expression).

PHA stimulation, six of the patients with SLE decreased their percentages of IL4-positive $\alpha\beta^+$ cells after stimulation.

Quantitation of $\alpha\beta^+$ double-negative T cells

Our previous work had indicated that the $\alpha\beta^+$ DN T cell subpopulation was expanded within the total DN population in patients with SLE.[3] While the total lymphocyte population comprised approximately 1–12% DN T cells in all experimental groups, an average of 60% of these cells were $\alpha\beta^+$ in patients with SLE compared with an average of 48% in healthy people ($P = 0.0216$). Comparison of percentages of $\alpha\beta^+$ DN T cells as a proportion of total $\alpha\beta^+$ T cells for those 15 patients who had the highest frequency of IL4-positive $\alpha\beta^+$ DN T cells with percentage of $\alpha\beta^+$ DN T cells for those patients with low frequency of IL4-positive cells, showed that those patients with high percentages of IL4-positive cells

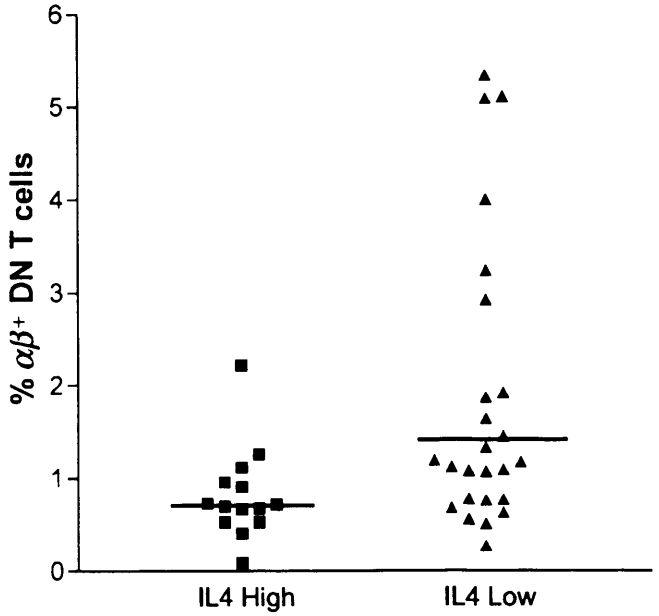


Figure 3 Patients with the highest percentages of IL4-containing $\alpha\beta^+$ DN T cells had the lowest levels of $\alpha\beta^+$DN T cells. Patients were grouped into those with greater than 2 s.d. from the mean percentages of constitutive IL4 containing T cells of the patients with SLE (IL4 high) and those with percentages of IL4-containing T cells less than 2 s.d. from the mean (IL4 low).

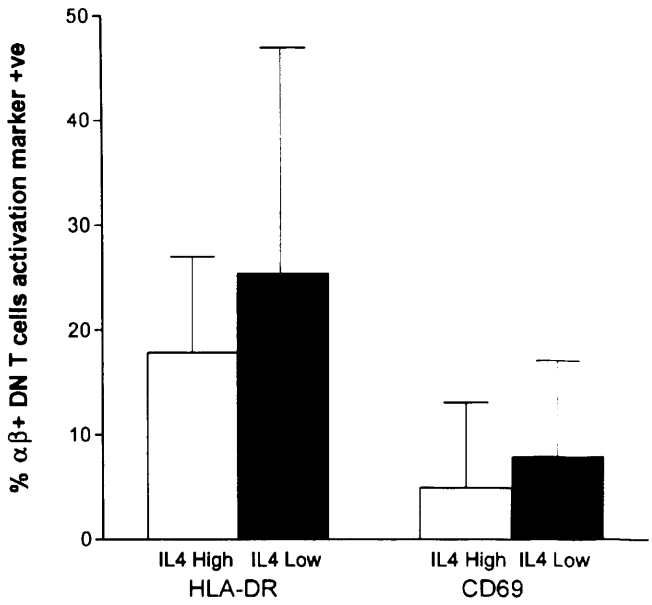


Figure 4 There is no relationship between activation marker expression and high and low IL4 expression by $\alpha\beta^+$ DN T cells in patients with SLE. PBMCs from patients with SLE were stained for expression of HLA-DR and CD69 as described in the Materials and methods and grouped into constitutive high and low IL4 producers as described in Figure 3.

had significantly fewer $\alpha\beta^+$ T cells in the DN compartment than did the group with low frequency of IL4-positive cells ($P=0.02$; Figure 3). (Further analysis showed that there was no overall correlation between percentages of IL4-positive $\alpha\beta^+$DN T cells and total $\alpha\beta^+$DN T cells ($r=-0.15$, $P=0.36$)).

No correlation of IL4 production with cell surface markers

Levels of the surface activation markers CD69 and HLA-DR were measured on $\alpha\beta^+$DN T cells from 21 patients with SLE, 17 of whom had low frequencies of IL4-positive cells and four had high frequencies of IL4 positive $\alpha\beta^+$DN T cells. There was no significant difference in activation marker expression between high and low producers of IL4 ($P=0.51$ for HLA-DR and $P=0.58$ for CD69; Figure 4). In addition, there was no significant correlation between expression of activation markers and high or low frequency of IL4-positive $\alpha\beta^+$DN T cells ($r=-0.12$, $P=0.33$ for HLA-DR and $r=0.78$, $P=0.39$ for CD69; Figure 4). In the above experiments, DN T cells were independently evaluated for activation markers and IL4 expression. It was not possible to determine whether the IL4 containing cells themselves expressed HLA-DR or CD69 in this study owing to the technical limitations of three-colour staining.

No correlation between constitutive IL4 production and disease activity

There was no correlation between the BILAG Global Disease Activity Index and IL4 production ($r=0.07$, $P=0.6$). Similarly, when the patients with SLE were divided into groups depending on the specific organs or systems affected, there was no connection between IL4 production and the organ or system involved. It was not possible to calculate correlation coefficients for each of the specific organs or systems owing to the small sample size.

No association between constitutive IL4 production and disease duration

The 12 patients in the high constitutive IL4 group had a mean disease duration of 10.67 ± 2.64 years. The low IL4 group consisted of 29 patients of mean disease duration 12.64 ± 1.56 years. There was no significant difference in disease duration between the two groups ($P=0.6$).

No association of constitutive IL4 production and drug treatment

All but one of the patients with SLE who showed high levels of IL4-positive $\alpha\beta^+$DN T cells was receiving prednisolone therapy (dosage varying from 3 to 10 mg). Five of these patients were also being treated with Azathioprine (dosage varying from 50 to 100 mg).

The remaining patient was being treated with antimalarial drugs (hydroxychloroquine, 400 mg). Twenty-four of the low-IL4 group were also being treated with prednisolone, 11 of these in combination with azathioprine and one in combination with antimalarials. One patient was receiving azathioprine alone, seven were receiving antimalarials alone, and one patient was being treated with a combination of antimalarials and azathioprine. There was no association between drug treatment and levels of constitutive production of IL4. When the patients treated with prednisolone were divided arbitrarily on the basis of steroid dosage into high and low dose groups (≥ 10 and < 10 mg, respectively), no significant differences in percentages of IL4-positive $\alpha\beta^+$DN T cells or percentages of $\alpha\beta^+$DN T between the two groups were found ($P = 0.77$ and $P = 0.84$, respectively).

No correlation between IL4 production and plasma C3

Plasma C3 is measured routinely in all patients attending the Lupus clinic. Low levels of C3 often correlate with active disease. However, in 47 patients for whom data were available, percentages of $\alpha\beta^+$DN T cells constitutively expressing IL4 did not correlate with plasma C3 ($r = 0.21$, $P = 0.44$).

No correlation between IL4 production and circulating anti-dsDNA antibodies

Circulating anti-dsDNA antibodies are a characteristic of SLE and high levels of antibody are a predictor of severe disease. In the 47 patients for whom data were available, there was no correlation between levels of circulating anti-dsDNA antibodies and the percentages of IL4-positive $\alpha\beta^+$DN T cells ($r = 0.07$ $P = 0.66$). However, there was a statistically significant negative correlation between C3 and anti-dsDNA for the patient sample considered in this study ($r = -0.45$, $P = 0.01$), confirming the known relationship between high anti-dsDNA antibodies and low C3.

No association of constitutive IL4 production with ethnic background

Of the 12 patients with SLE showing elevated frequency of constitutive IL4-positive $\alpha\beta^+$DN T cells, nine were Caucasian, one Indian, one Afro-Caribbean and one Chinese. All of these patients were female. Twenty-four of the group with a low frequency of IL4 positive cells were Caucasian, four Indian, one Afro-Caribbean, one mixed race Caucasian and Afro-Caribbean and two Chinese; one of these patients was male. There was no association between ethnic background and levels of IL4 production.

No association of IL4 production with HLA phenotype

Tissue-typing data were not available for all of the patients but were obtained for nine of the high-frequency IL4-positive group and for 24 of the low-frequency group. There was a wide variation in HLA expression, but it was noted that several phenotypes occurred multiple times. Five of the 24 patients in the low-frequency group expressed HLA A1 and HLA B8 while another five expressed HLA DQ5. In the high-frequency group, four of the nine patients expressed each of these phenotypes. Fischer's exact test indicated that the differences in expression between the two groups were not significantly different ($P = > 0.5$) as might be expected, given the relatively small sample number.

Discussion

In this study we have shown that the $\alpha\beta^+$DN T cell compartment of patients with SLE contains more cells positive for intracellular IL4 than the equivalent cells of RA autoimmune controls or healthy controls. Our results are consistent with the findings of other groups who have reported increased production of IL4 by T cells from patients with autoimmune and atopic diseases. Since we only looked at IL4 and not IFNγ in our study we were unable to determine whether IL4 producers were also IFNγ^+. Funauchi *et al.* have demonstrated that the frequency of IL4-positive cells is increased in SLE compared with interferon gamma (IFNγ) producers[12] and it has been shown that IL4 enhances differentiation of not only B cells but also T cells, particularly Th2 helper T cells.[13] A higher frequency of NK T cells (a subpopulation of $\alpha\beta^+$DN T cells) producing IL4 in patients with SLE than controls, following stimulation with PMA and Ionomycin has been described.[14] This NKT population mainly utilizes Vα24JαQVβ11 + T cell receptor in healthy subjects and these cells have been shown to produce mostly IFNγ on activation. Then, production of IFNγ by these cells would counterbalance the action of IL4 in augmenting the differentiation of naive T cells into Th2 cells. This population of Vα24Jα QVβ11$^+$DN T cells has been shown by others to be reduced in patients with active SLE[6] and to have dysfunction in about 50% of patients,[15] although DN Vα24$^+$ T cells are expanded overall. When these patients become inactive with the help of prednisolone therapy, Vα24JαQVβ11$^+$DN T cells become detectable once again, but it is unclear as to whether the function is restored. This finding would implicate Vα24JαQVβ11$^+$DN T cells in control of the pathogenesis of SLE possibly through the production of IFNγ.

It is unclear at present why only a minority of patients had an increased frequency of intracellular IL4 in the $\alpha\beta^+$ DN subpopulation, suggesting that these patients are in some way different from others within the group. In addition, a high frequency of intracellular IL4 appeared to be specific to the $\alpha\beta^+$ DN T cell population and was not found in the $CD4^+$ and $CD8^+$ populations. Attempts to correlate the increased expression of IL4 by the $\alpha\beta^+$ DN T cell population with a variety of other criteria failed to yield any relationships. These patients were not from a common ethnic background, did not share a common HLA phenotype and there was no relationship between the severity of their disease at the time of sampling and the percentage of IL4-positive cells. This latter observation is in contrast to the work of another group,[14] who found that disease activity was lowest in those patients who produced the highest levels of IL4. Indeed, the highest levels of IL4 in their patient cohort were produced by patients with a SLEDAI (systemic lupus erythematosus disease activity index[9]) GS of one. However, their data were generated from only the subpopulation of DN cells expressing CD57 not from the whole $\alpha\beta^+$ DN T cell population and so may not be comparable with our findings.[14] There appeared to be no relationship between levels of plasma C3 and anti dsDNA antibodies and IL4 produced, suggesting that the high levels of intracellular IL4 in some patients are not simply related to disease activity using these criteria. In addition, there appeared to be no obvious relationship between drug therapy and production of IL4. Interestingly, almost all of our patients were being treated with prednisolone.

Further analysis of the data showed that the percentages of $\alpha\beta^+$ DN T cells present in the 15 patients with the highest frequency of IL4 containing cells was significantly lower than that in the other patients with SLE. Although the significance of this observation is not yet clear, it may lend weight to the theory that high levels of IL4 within the DN T cell population are seen in those patients who lack a regulatory population of cells that would otherwise control cytokine production. Alternatively, it is possible that when the $\alpha\beta^+$ DN T cells are present in lower numbers, they receive more help to produce cytokines. Such a possibility would suggest that the IL4-producing cells would be more activated and measurement of activation markers on the whole subpopulation of $\alpha\beta^+$ DN T cells appeared to confirm this theory, although the difference was not significant and numbers were too low to draw firm conclusions. These findings are consistent with those of our previous study, showing that $\alpha\beta^+$ DN T cells express higher than normal levels of CD69 and HLA-DR.[3] However, it was not possible to determine whether those cells positive for intracellular IL4 were also activated within the constraints of three-colour staining and further work would be needed to clarify this point. Since IL4 is under genetic control and polymorphisms have been shown to be related to its production,[15,16] it would be valuable in future studies to determine any differences in the genotype in relation to IL4 production. It is possible that the increased receptor function seen in some patients with SLE may play a role in the development of disease through induction of Th2 subset development. The observation that stimulation with PHA masked the differences observed between the experimental groups may be explained by the fact that cells in patients with SLE are already in an activated state and have been widely shown not to react strongly to mitogenic stimulation.[18] Stimulation with PHA caused most of the individuals tested to have a higher percentage of IL4-positive $\alpha\beta^+$ DN T cells although there appeared to be a lower level of stimulation in the patients with SLE. Indeed, six of these patients could be seen to have a lower percentage of IL4 positive DN T cells than they expressed constitutively. It is unclear as to why these six patients had reduced levels of IL4-containing cells since there was no apparent correlation following PHA stimulation with disease activity, disease duration or percentage of $\alpha\beta^+$ DN T cells. In addition, these six patients were not concordant for drug treatment and did not appear to be concordant for HLA phenotype.

In conclusion, our data indicate that in about one third of patients with SLE, there is a higher frequency of $\alpha\beta^+$ DN T cells that constitutively express IL4 than in control individuals. This increase in IL4 was more accentuated in the $\alpha\beta^+$ DN T cells than in the $CD4^+$ and $CD8^+$ T cell populations, suggesting that the DN T cell compartment could be functionally more important than the conventional $\alpha\beta^+$ T cells in these patients with SLE. Although there was no direct correlation with anti DNA antibodies, or indeed with other parameters such as disease activity or drug treatment, it is possible that this population could contribute to autoantibody production but other populations not measured could be regulating the function of the $\alpha\beta^+$ DN T cells in SLE.

Acknowledgements

This work was funded by a grant from the Arthritis Research Campaign.

References

1 Isenberg D, Horsfall A. Systemic lupus erythematosus. In: Moodison PJ, Isenberg DA, Glass D, Woo P (eds). *Oxford Textbook of Rheumatology*. Oxford University Press: Oxford, 1998, pp 1145–1180.

2 Murison JG, Quaratino S, Kahan M, Verhoef A, Londei M. Definition of unique traits of human CD4-CD8- alpha beta T cells. *Clin Exp Immunol* 1993; **93**: 464–470.
3 Anand A, Dean G, Querishi K, Isenberg D, Lydyard P. Characterisation of $CD3^+$, $CD4^-$, $CD8^-$ (double negative) T cells in patients with systemic lupus erythematosus (SLE). *Lupus* 2002; **11**: 493–500.
4 Sumida T, Sakamoto A, Murata H *et al.* Selective reduction of T cells bearing invariant V alpha 24J alpha Q antigen receptor in patients with systemic sclerosis. *J Exp Med* 1995; **182**: 1163–1168.
5 Wilson SB, Kent SC, Patton KT *et al.* Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. [Published erratum appears in *Nature* 1999; **399**(6731): 84.] *Nature* 1998; **391**: 177–181.
6 Oishi Y, Sumida T, Sakamoto A *et al.* Selective reduction and recovery of invariant Valpha24JalphaQ T cell receptor T cells in correlation with disease activity in patients with systemic lupus erythematosus. *J Rheumatol* 2001; **28**: 275–283.
7 Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271–1277.
8 Hay EM, Bacon PA, Gordon C *et al.* The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. *Q J Med* 1993; **86**: 447–458.
9 Gladman DD, Goldsmith CH, Urowitz MB *et al.* Sensitivity to change of 3 Systemic Lupus Erythematosus Disease Activity Indices: international validation. *J Rheumatol* 1994; **21**: 1468–1471.
10 Arnett FC, Edworthy SM, Bloch DA *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; **31**: 315–324.
11 Dinter A, Berger EG. Golgi-disturbing agents. *Histochem Cell Biol* 1998; **109**: 571.
12 Funauchi M, Ikoma S, Enomoto H, Horiuchi A. Decreased Th1-like and increased Th2-like cells in systemic lupus erythematosus. *Scand J Rheumatol* 1998; **27**: 219–224.
13 Ferdinandez BR, Sanders VM, Mosmann TR, Vietta ES. Lymphokine-mediated regulation of the proliferative response of clones of T helper and T helper 2 cells. *J Exp Med* 1988; **168**: 543–558.
14 Funauchi M, Yu H, Sugiyama M *et al.* Increased interleukin-4 production by NK T cells in systemic lupus erythematosus. *Clin Immunol* 1999; **92**: 197–202.
15 Kojo S, Adachi Y, Keino H *et al.* Dysfunction of T cell receptor AV24AJ18+, BV11+ double-negative regulatory natural killer T cells in autoimmune diseases. *Arthritis Rheum* 2001; **44**: 1127–1138.
16 Klein W, Tromm A, Griga T *et al.* Interleukin-4 and interleukin-4 receptor gene polymorphisms in inflammatory bowel diseases. *Genes Immunol* 2001; **2**: 287–289.
17 Kanemitsu S, Takabayashi A, Sasaki Y *et al.* Association of interleukin-4 receptor and interleukin-4 promoter gene polymorphisms with systemic lupus erythematosus. *Arthritis Rheum* 1999; **42**: 1298–1299.
18 Hernandez-Fuentes MP, Reyes E, Prieto A *et al.* Defective proliferative response of T lymphocytes from patients with inactive systemic lupus erythematosus. *J Rheumatol* 1999; **26**: 1518–1526.